VIRULENCE GENE DETECTION AND EXPRESSION IN *STREPTOCOCCUS DYSGALACTIAE* SUBSP. *DYSGALACTIAE* STRAINS AND EVALUATION OF INFECTION POTENTIAL

JOÃO MANUEL MATA CAÇO

DISSERTATION SUBMITTED FOR THE DEGREE OF MASTER IN MEDICAL MICROBIOLOGY

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Abstract

Streptococcus dysgalactiae subsp. *dysgalactiae* (SDSD) is considered an exclusively animal pathogen and *Streptococcus pyogenes* (GAS) a strictly human pathogen. GAS phage virulence determinants were recently found in SDSD strains of bovine origin, and cases of human infection associated with SDSD have been recently reported. The SDSD zoonotic potential has been therefore suggested, however the role of those virulence genes in the pathogenesis of the bovine SDSD has not been proved.

One of the objectives of this thesis was to detect the presence and expression of GAS virulence determinants, among contemporary SDSD strains, isolated from milk samples of bovines diagnosed with mastitis in Portuguese dairy herds between 2011-13 and compare the data with the one previously reported of a study of a Portuguese SDSD collection of 2002-03. *In vitro* and *in vivo* infection potential was also evaluated and compared between both collections. GAS genetic determinants (virulence genes *speB*, *speC*, *speF*, *speH*, *speK*, *speL*, *speM*, *smeZ*, *spd1*, *sdn* and the chimeric element *Tn1207.3/Φ10394.4*) were screened by PCR and their expression was assessed by PCR after cDNA synthesis. Extracellular DNase production was assessed and correlated with *spd1* and *sdn* genotypic profile. To study the infection potential, *in vitro*, human normal and tumoral respiratory cell lines (BTEC and Detroit 562, respectively) were used, and *in vivo*, the zebrafish animal model was chosen. Results suggested that the virulence determinants screened are characteristic of SDSD of bovine origin and that extracellular DNase production was independent on the *spd1* and *sdn* genes. *In vitro* and *in vivo* infection studies revealed that the infection potentials of SDSD are strain-specific and independent on the virulence genes screened. Zoonotic potential of SDSD is further suggested, as strains from bovine origin were able to infect human cell lines, as well as the zebrafish.

Keywords: *Streptococcus dysgalactiae* subsp. *dysgalactiae*, *Streptococcus pyogenes*, zoonosis, virulence genes, human respiratory cell lines, zebrafish.

Resumo

Streptococcus dysgalactiae subsp. *dysgalactiae* (SDSD) é considerado um agente patogénico animal exclusivo e *Streptococcus pyogenes* (GAS) um agente patogénico humano exclusivo. Recentemente foram encontrados fatores de virulência fágicos de GAS em estirpes de SDSD de origem bovina e casos de infeção humana associada a SDSD têm vindo a ser reportados. Em consequência, o potencial zoonótico de SDSD foi sugerido, contudo o papel destes fatores de virulência na patogénese de SDSD não foi comprovado.

Um dos objetivos desta tese foi detetar a presença e expressão de fatores de virulência de GAS, entre isolados de SDSD contemporâneos de origem portuguesa, isolados de amostras de leite de bovinos disgnosticados com mastite em herdades leiteiras portuguesas entre 2011-13 e comparar estes dados com os reportados de uma coleção portuguesa de SDSD previamente estudada de 2002-03. O potencial de infeção *in vitro* e *in vivo* foi também avaliado e comparado entre coleções. Determinantes genéticos de GAS (os genes de virulência *speB*, *speC*, *speF*, *speH*, *speK*, *speL*, *speM*, *smeZ*, *spd1*, *sdn* e o elemento quimérico *Tn1207.3/Φ10394.4*) foram pesquisados por PCR e a sua expressão averiguada por PCR após síntese de cDNA. A produção de DNases extracelulares foi avaliada e correlacionada com o perfil genotipico dos genes *spd1* e *sdn*. Para estudar o potencial de infeção, *in vitro*, foram utilizadas linhas celulares repiratórias normais e tumorais humanas (BTEC e Detroit 562, respetivamente) e *in vivo*, o modelo animal zebrafish. Os resultados sugerem que os fatores de virulência pesquisados são característicos de SDSD de origem bovina e a produção de DNases extracelulares é independente dos genes *spd1* e *sdn*. Os estudos de infeção *in vitro* e *in vivo* revelam que os potenciais de infeção de SDSD são específicos de estirpe e independentes dos genes de virulência pesquisados. O potencial zoonótico de SDSD é novamente sugerido uma vez que estirpes de origem bovina foram capazes de infetar linhas celulares humanas e o zebrafish.

Palavras-chave: *Streptococcus dysgalactiae* subsp. *dysgalactiae*, *Streptococcus pyogenes*, zoonose, genes de virulência, linhas celulares respiratórias humanas, zebrafish.

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1. Introduction

1.1. The *Streptococcus* genus

1.1.1. Main Characteristics and Overview

Bacteria from the *Streptococcus* genus are gram-positive, spherical or ovoid shaped (cocci) in pairs or chains and do not have motility neither the capability to form spores (Hardie and Whiley, 1997). They are also catalase negative and facultative anaerobic, with some requiring atmospheric CO_2, being homofermentative producing L-lactic acid as their main product of glucose fermentation (Hardie and Whiley, 1997).

It is important to differentiate the different streptococcal species since their distribution and health implications are broad (Facklam, 2002; Hardie and Whiley 1997). Generally, streptococcal species are mainly present as flora of human and animal mucosal surfaces, such as the gastrointestinal and upper respiratory tracts, and skin (Krzyściak *et al*., 2013). Some species associated with humans and/or animals can pose a threat to their health since they might become opportunistic pathogens and cause an array of diseases in individuals with weak immune systems (Krzyściak *et al*., 2013).

Other *Streptococcus* species that are typically pathogenic to humans are *Streptococcus pyogenes*, the most pathogenic bacteria of the genus, and *Streptococcus pneumonia* (Krzyściak *et al*., 2013). The former known for causing pharyngitis, impetigo and severe invasive diseases, and the latter for community-acquired pneumonia and meningitis among other diseases (Cunningham, 2000; Facklam *et al*., 2002). As for known animal pathogens, *Streptococcus agalactiae*, *Streptococcus uberis* and *Streptococcus dysgalactiae* subsp. *dysgalactiae* are well described as being associated with bovine mastitis (Lundberg *et al*., 2014).

1.1.2. Haemolysis

Streptococcal haemolysis is one of the main phenotypic characteristics which, in coordination with other methods, help identify the different species of the genus based on haemolytic reactions on blood agar medium.

β-haemolysis is characterized by a clear translucent halo surrounding *in vitro* colonies on blood agar plates and can be used as the preliminary identification method for *S. pyogenes* (Molloy *et al*., 2014). Streptolysins, as other cholesterol-dependant cytolysins, are toxins secreted by the bacterial cells that bind to cholesterol-containing cell membranes, such as erythrocytes, and oligomerize to create pores by insertion into the lipid bilayer leading to osmotic cell lysis of the host (Barnett *et al*., 2015; Molloy *et al*., 2014).

Another type of haemolysis, α-haemolysis, is characterized by a green halo surrounding *in vitro* colonies on blood agar plates (Facklam, 2002). This type of haemolysis can also be used for presumptive identification of species of the *Streptococcus* genus, such as *Streptococcus dysgalactiae* subsp. *dysgalactiae*, relies on partial erythrocyte haemolysis (Facklam, 2002). Other streptococcal species incapable of producing haemolysis on blood agar plates are said to be γ-hemolytic or non-hemolytic (Facklam, 2002; Patterson, 1996).

1.1.3. Cell Wall Carbohydrate Composition

Streptococcal species are frequently differentiated on the basis of their cell wall carbohydrate composition through a classification method based on the precipitin test developed by Lancefield (1933). This classical classification method is possible due to the existence of different group carbohydrate antigens composed of one or more sugars which, after extraction from the cell-wall, and by agglutination with the appropriate antiserum, causes the visual precipitation of the cell extract (Facklam *et al*., 2002; Lancefield, 1933; Slade and Slamp, 1962).

There are 17 different serological groups spanning from A-H and K-S, with rhamnose being present in antigens of strains in all groups with the exception of group O

Streptococcus (Slade and Slamp, 1962). It is known that group A *Streptococcus* (GAS) and group C *Streptococcus* (GCS) strains have group carbohydrate antigens mainly composed of rhamnose (Slade and Slamp, 1962). Nowadays there are commercially available Lancefield grouping kits that permit rapid grouping of Streptococci by polystyrene (latex) microparticle agglutination, coated with group specific antibodies, when challenged with the corresponding cell-wall antigen extract (Facklam *et al.*, 1979; Lue *et al.*, 1978).

1.1.4. Molecular Identification and Streptococcal Grouping

Throughout the years some of the most used molecular identification methods for streptococcal genera and species have been DNA-DNA hybridization and small subunit (16S) rRNA sequencing (Facklam, 2002). The latter method is based on the sequencing and alignment comparison of portions of the 16S rRNA gene and has helped define 6 different main streptococcal groups; the Pyogenic, Anginosus, Mitis, Salivarius, Bovis and Mutans (Bentley *et al.*, 1991; Kawamura *et al.*, 1995). Some authors suggest a seventh group with species that do not fit in any of the previous defined groups (Gao *et al.*, 2014).

The pyogenic group comprises species isolated from humans and other animals (Facklam, 2002). This group is very broad as there are human pathogens such as *S. pyogenes* and *Streptococcus dysgalactiae* subsp. *equisimilis* (also an animal pathogen), and animal pathogens such as *S. agalactiae* (also a human pathogen), *Streptococcus dysgalactiae* subsp. *dysgalactiae* and *Streptococcus uberis* (Facklam, 2002). The Anginosus group comprises species isolated from the human urogenital and gastrointestinal tracts, such as *Streptococcus anginosus*, and only from the respiratory tract such as *Streptococcus constelatus* subsp. *constelatus* (Facklam, 2002). Lancefield grouping of these species is difficult as there are strains from both species with group A, C, F and G carbohydrate antigens and even non-groupable (Facklam, 2002). The Mitis group includes several known streptococcal species isolated from the human respiratory tract, mainly the oral cavity (Facklam, 2002). This group comprises *Streptococcus mitis*, a human commensal organism of the oropharynx that can become opportunistic, and *Streptococcus pneumoniae*, a human colonizing pathogen of the upper respiratory tract

such as the naso-oropharynx and responsible for acute otitis media and pneumonia (Facklam, 2002; Gossling, 1988; Kadioglu *et al.*, 2008; Mitchell, 2010). The Salivarius group comprises species isolated from the oral cavity of humans and animals (Facklam, 2002). The Bovis group contains species found in animals and humans, with *Streptococcus bovis* being present in the gut and causing endocarditis, gastrointestinal diseases and being implied in colon cancer (Galdy and Nastasi, 2012). The Mutans group comprises species found in the oral cavity of humans and other animals and include *Streptococcus mutans* which is known to lead to human caries (Facklam, 2002). Table 1 shows an overview of the six defined groups and its most important species with their main characteristics (Barnard and Stinson, 1996; Bentley *et al.*, 1993; Bramley, 1984; Facklam 1974; Facklam, 2002; Grinwis *et al.*, 2010; Kadioglu *et al.*, 2008; Kilian *et al.*, 1989; Ruoff *et al.*, 1984).

Table 1. Streptococcal grouping based on 16S rRNA sequencing.

Group	Species	Lancefield group	Haemolysis	Origin
Pyogenic	*S. pyogenes*	A	β-	Human
	S. agalactiae	B	β-	Human, bovine
	S. dysgalactiae			
	subsp. *dysgalactiae*	C	α-	Animals
	subsp. *equisimilis*	A, C, G, L	β-	Human, animal
	S. uberis	E, C, D, P, U, N/G	γ-	Bovine
	S. equi			
	subsp. *equi*	C	β-	Horse
	subsp. *zooepidemicus*	C	β-	Human, animals
Anginosus	*S. anginosus*	A, C, F, G, N/G	β-, N/ β-	Human
	S. constellatus			
	subsp. *pharyngis*	C	β-	Human
Mitis	*S. pneumoniae*	N/G	α-	Human
	S. mitis	O, K	α-	Human
	S. gordonii	H	α-	Human
Salivarius	*S. salivarius*	N/G	γ-	Human
Bovis	*S. bovis*	D	γ-	Human, animals
Mutans	*S. mutans*	E, F, K, N/G	α-, β-, γ-	Human

N/G – non-groupable; N/ β – non-β-haemolytic

1.2. Streptococcal Pathogenesis

1.2.1. *S. pyogenes* as a Human Pathogen

As the most pathogenic bacteria of the *Streptococcus* genus, the β-hemolytic group A *S. pyogenes* (GAS) can cause a variety of severe diseases (Lamagni *et al*., 2008). *S. pyogenes* can colonize the throat and skin of humans, being the most common cause of bacterial pharyngitis, the causal agent of impetigo and scarlet fever, and acute rheumatic fever (Barnett *et al*., 2015; Cunningham, 2000; Facklam, 2002). Primary focal sites of infection are precisely these tissues which are also the primary reservoirs of transmission (Efstratiou, 2000). Diseases caused by this pathogen vary from superficial infections such as pharyngitis, skin and soft tissue infections and erysipelas to more severe infections such as deep soft tissue infections, cellulites, necrotizing fasciitis, sepsis, pneumonia or meningitis (with the possibility of fatal consequences for the latter three). Moreover, toxin-mediated diseases such as scarlet fever and toxic-shock syndrome, and immunologically mediated diseases such as rheumatic fever and post-streptococcal glomerulonephritis (which can also be toxin-mediated) are also caused by *S. pyogenes* (Barnett *et al*., 2015; Cunningham, 2000; Efstratiou, 2000).

Figure 1. Patient with group A streptococcal toxic shock syndrome and necrotizing fasciitis. Adapted from Johansson *et al*. (2010).

The severity and type of disease depends on the afflicted tissue, may it be mucous membranes, tonsils, skin or deeper tissues, immunological state of the carrier and the bacteria itself, as it can carry a variety of virulence factors responsible for its pathogenicity (Barnett *et al*., 2015; Cunningham, 2000). Development of post-streptococcal infection sequelae include rheumatic fever, acute glomerulonephritis and reactive arthritis, with the former being the most serious autoimmune sequelae of *S. pyogenes* even causing death (Cunningham, 2000).

1.2.1.1. *S. pyogenes* Virulence Factors and Phage Encoded Genes

Many of the previously mentioned diseases caused by *S. pyogenes* pathogenesis are due to the existence of several virulence factors (Barnett *et al*., 2015). As stated above, toxic-shock and invasion of soft tissues and skin, and necrotizing fasciitis are examples of this.

Virulence factors such as the streptococcal pyrogenic exotoxins and other superantigens, DNases and streptodornases, and adhesins are well known factors responsible for *S. pyogenes* pathogenicity (Steer *et al*., 2012).

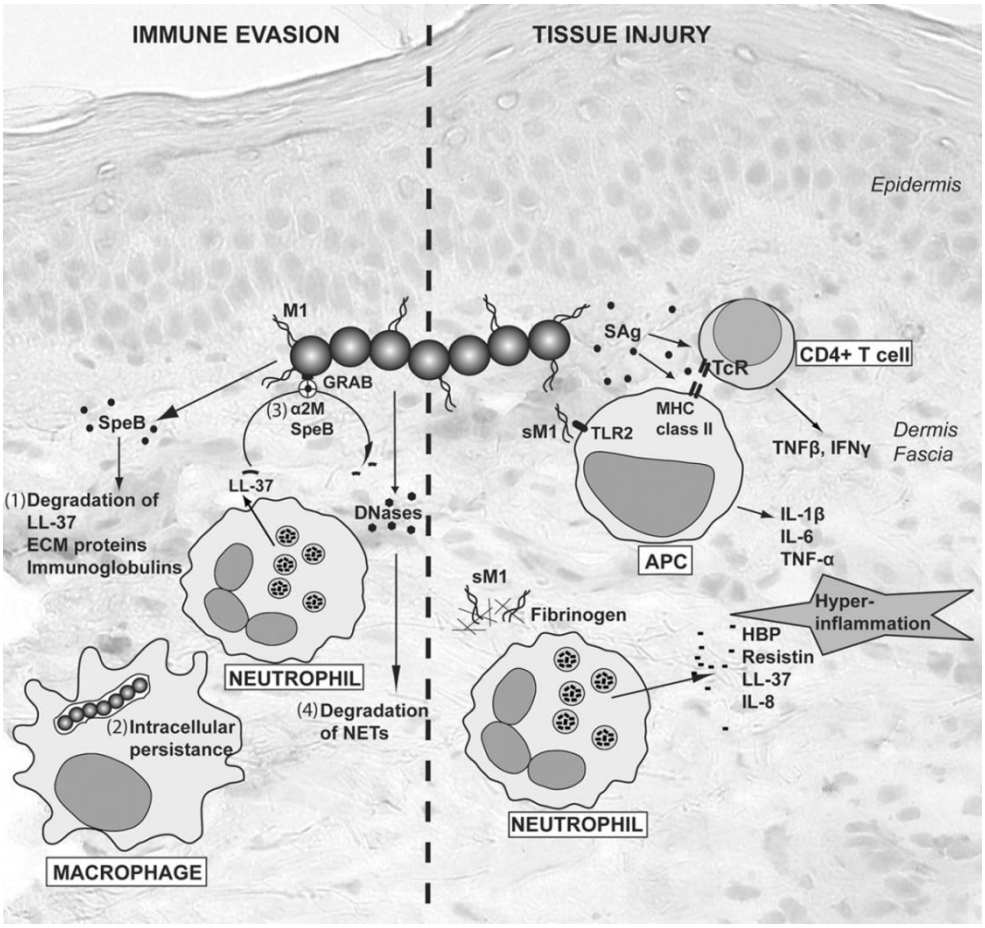

Figure 2. Interactions at the tissue site between group A *Streptococcus pyogenes* and host during severe deep-tissue infections. Mechanisms involved in microbial immune evasion: 1 – Degradation of host immune effector molecules; 2 – Intracellular persistence within phagocytic cells; 3 – Protection against antimicrobial molecules; 4 – Degradation of neuthrophil extracellular traps (NETs) by extracellular DNases. Mechanisms involved in host tissue injury: Degradation of ECM proteins by SpeB; Induction of an excessive inflamatory response through the activation of T cells, neuthrophils and antigen presenting cells (APCs) mediated by superantigens (Sags) and soluble M1 protein (sM1). Adapted from Johansson *et al.* (2010).

1.2.1.1.1. Extracellular DNases

Many species of bacteria have the ability to synthesise extracellular deoxyribonucleases (DNases) which can be anchored to its surface or secreted (Jakubovics *et al.*, 2013). These enzymes may have a variety of applications.

One of the characteristics of this type of DNases is the bacteria ability to control biofilm growth since extracellular DNA is a major structural component of microbial biofilms (Jakubovics *et al.*, 2013). Microbial extracellular nucleases also help other

microorganisms to find sources of carbon, nitrogen and phosphorous in released nucleotides from degraded extracellular DNA (Jakubovics *et al.*, 2013). EndA, a cell anchored DNase, has the ability to nick extracellular double-stranded DNA into single-stranded for uptake during bacterial transformation (Jakubovics *et al.*, 2013).

But perhaps the most important characteristic of extracellular DNases in bacterial pathogenesis is helping with evasion from the innate immune system of the host (Brinkman *et al.*, 2004). During an inflammatory infection neuthrophils migrate from the blood to the infected tissues and can either phagocyte the pathogen or release extracellular nets, termed Neutrophil Extracellular Traps (NETs) which are independent from the phagocytic uptake (Brinkmann *et al.*, 2004; Buchanan *et al.*, 2006). With a fibrous structure, NETs are composed of granule and nuclear constituents such as proteins and DNA, being the former its major structural component (Hahn *et al*., 2013). These traps can capture, preventing microbe spreading, and kill bacteria upon delivery of antimicrobial molecules and can also degrade their virulence factors (Brinkmann *et al.*, 2004). To evade this immune mechanism and maintain spreading through other tissues some bacteria, such as *S. pyogenes* and *S. pneumoniae*, synthesize extracellular DNases. Streptococcal DNases are referred to as streptodornases (Aziz *et al*., 2004).

Evasion of *S. pneumoniae* relies on the aforementioned enzyme, EndA (Beiter *et al.*, 2006). As for *S. pyogenes*, it is known that all strains produce at least one and most isolates can produce two or more DNases (Ferreira *et al*., 1992; Sumby *et al*., 2005). These are DNase A (Spd3), DNase B (SdaB), DNase C (Spd1) and DNase D (SdaD) with 4 known homologs for the latter (Sda, Sda1, Sdn and Sdα). All except DNase B are phage-encoded. In *S. pyogenes* M1T1 strain, synthesis of Sda1 is essential for NET evasion (Aziz *et al.*, 2004; Buchanan *et al.*, 2006).

Spd1, formerly known as DNase C, is a monomeric ββα-metal dependant endonuclease encoded by the *spd1* gene located, in linkage with *speC*, on multiple phage genomes such as Φ370.1, Φ10750.1, Φ8232.2, Φ6180.1, Φ10270.1, man.4, Φ10394.5, Φ9429.1 and Φ2096.1.(Beres and Musser 2007; Korczynska *et al*., 2012). Once the target DNA or RNA contacts the binding site within the structure of Spd1, its nucleotide 5'-phosphate is cleaved in a non-specific manner (Korczynska *et al*., 2012).

1.2.1.1.2. Streptococcal Superantigens

Streptococcal superantigens are a class of the Gram-positive bacterial superantigens (SAgs) that can lead to life-threatening systemic disease by toxic shock syndrome, and scarlet fever (Fraser and Proft, 2008; Okumura *et al.*, 2012). With eleven known streptococcal superantigens spread throughout *S. pyogenes* genomes, they act as crosslinkers between the histocompatibility complex class II (MHC-II) and T_{CD4}-cell receptors (TCR) causing the stimulation of T-cells and increased cytokine secretion, such as IL-2, IFN-γ and TNF-α (Barnett *et al*., 2015; Okumura *et al.*, 2012). After the binding of SAgs to MHC-II, it concentrates stably onto the antigen presenting cell surface (such as B-cells, monocytes and dendritic cells) until its surface concentration is sufficient to successively engage and cross-link multiple TCR molecules, resulting in strong TCR signalling and rapid cytokine production (Barnett *et al*., 2015; Fraser and Proft, 2008).

SAgs such as the streptococcal pyrogenic exotoxins C (encoded by the *speC* gene), H (*speH*), K (*speK*), L (*speL*), M (*speM*) and the streptococcal mitogenic exotoxin Z (*smeZ*) have a zinc binding site, and bind to the MHC-II β-chain domain and to the TCR variable region of the β-chain (Vβ) (Barnett *et al*., 2015; Fraser and Proft, 2008).
As termed in literature, SpeB, encoded by *speB*, does not act as a superantigen itself, being instead a multifunctional cysteine protease that regulates other SAgs at the protein level through proteolysis, and degrades immunoglobulins (*in vitro*), complement components and host extracellular matrix proteins potentially resulting in deeper tissue damage (Barnett *et al*., 2015; Fraser and Proft, 2008). Another function of this molecule is its binding activity to laminin and other glycoproteins when anchored to the streptococcal surface acting as an adhesin (Hytönen *et al.*, 2001). Another interesting superantigen is SpeF, also having DNase activity and being characterized as the streptodornase SdaB (DNaseB) (Aziz et al., 2004; Eriksson et al., 1999).

The majority of these superantigens, particularly of *S. pyogenes*, are located mainly on prophages (bacteriophage genome inserted in the bacterial chromosome during the

lisogenic cycle), and in other genetic mobile elements (MGE) (Fraser and Proft, 2008) The role of MGE in streptococcal pathogenesis is discussed bellow.

1.2.1.1.3. Mobile Genetic Elements in *S. pyogenes* Pathogenesis

Mobile genetic elements (MGE) have an important role in streptococcal pathogenesis, in this particular case, in *S. pyogenes*. Many virulence factors such as superantigens, DNases, adhesins and even antibiotic resistance associated genes can be carried on numerous exogenous elements such as prophages, plasmids and pathogenicity islands, and can be transferred between different bacterial species by horizontal gene transfer (Pallen and Wren, 2007).These genetic elements are usually distinct from the bacterial chromosome in terms of nucleotide composition, mainly in their GC% content, indicating their exogenous nature from organisms not so closely related with the *Streptococcus* genus, and thus, generate genetic diversity (Beres and Musser, 2007; Reznikoff, 2003). This acquisition of genetic material provides an increased fitness for the recipient, acting as a selective advantage (Beres and Musser, 2007).

In *S. pyogenes* an example of an important MGE is the chimeric element *Tn1207.3/Φ10394.4*. Two important related genetic structures are the 52.5 kb conjugative transposon *Tn1207.3* (conjugative prophage by some authors), which has a complete copy of the *Tn1207.1* defective transposon found in *S. pneumoniae*, and the 58.8 kb *Φ10394.4* prophage, which also contains a complete copy of the same transposon (D'Ercole *et al.*, 2005; Iannelli *et al.*, 2014; Santagati *et al.*, 2003). Both carry the macrolide efflux pump *mef(A)* gene since it is present in *Tn1207.1* (Santagati *et al*., 2003). Furthermore, a third *tet(O)-mef(A)* structure, conferring both resistance to tetracycline and macrolides, is also related with *Tn1207.3/Φ10394.4* family (Brenciani *et al*., 2004). As stated above, other virulence genes such as *speC* (pyrogenic exotoxin C) and *spd1* (DNase1) can be found in linkage on the same prophage genome and be co-expressed upon bacteriophage induction (Beres and Musser 2007; Broudy *et al*., 2002). Gene linkage has also been reported in regards to *speL-speM* genes and associated with Φ8232.3 phage (Beres and Musser, 2007).

1.2.2. *S. dysgalactiae* subsp. *dysgalactiae* as an Animal Pathogen

As one of the most common isolated bovine pathogens, *Streptococcus dysgalactiae* subsp. *dysgalactiae* is an α-haemolytic group C *Streptococcus* (Abdelsalam *et al*., 2015; Lundberg *et al.*, 2014). Although mainly associated with bovine disease, such as mastitis, *S. dysgalactiae* subsp. *dysgalactiae* is also a known cause of ovine mastitis and an emerging fish pathogen (Abdelsalam *et al*., 2013; Lacasta *et al.*, 2008; Nomoto *et al.*, 2008). Other diseases such as bacteremia, meningoencephalitis and polyarthritis in sheep, polyarthritis in goats, and even neonatal death in dogs have also been reported (Lacasta *et al.*, 2008; Vela *et al.*, 2006).

1.2.2.1. Bovine Mastitis

Bovine mastitis is a disease characterized by the inflammation of the mammary gland (intramammary infection) and is the most devastating disease in terms of world economic losses related to dairy products (De Vliegher *et al.*, 2012; Seegers *et al.*, 2003). It can occur due to a number of varying microorganisms, depending geographically on the farm studied and including bacteria, namely *Streptococcus agalactiae*, *S. dysgalactiae* subsp. *dysgalactiae*, *Streptococcus uberis*, *Staphylococcus aureus*, Coagulase-negative staphylococci and even *Escherichia coli* (Bradley, 2002; Contreras and Rodríguez, 2011; Lundberg *et al.*, 2014; Waage *et al.*, 1999; Zadocks and Fitzpatrick, 2009).

Figure 3. Severe peracute mastitis in cattle caused by Klebsiella pneumoniae infection. Adapted from Ribeiro *et al*. (2008).

If the invading bacteria pass successfully through the anatomical barriers of the teat, such as the sphincter muscle and the keratinized epithelium, then it reaches the interior of the udder where the innate and acquired immune responses occur (Oviedo-Boyso *et al*., 2007). This can lead to infection and inflammation of the teat tissue (Oviedo-Boyso *et al*., 2007). The disease can then be distinguished between clinical and subclinical mastitis. The former being characterized by the presence of visible symptoms (abnormal milk, inflammation, etc.) and the latter by the visual absence of these (De Vliegher *et al.*, 2012).

Due to its clinical and subclinical manifestations, the disease may result in reduction of milk yield, which in turn is the main factor for the economic loss, and changes in milk composition related to a decrease in fats, lactose and casein, and increase in blood elements (serum albumin, immunoglobulins, somatic cells such as leukocytes and epithelial cells), chloride and sodium (Hortet and Seegers, 1998; Oviedo-Boyso *et al.*, 2007; Wellnitz and Bruckmaier, 2012). These effects place the farmers at a loss since less milk is sold and some is discarded due to altered composition (Blosser, 1979). Other factors that can cause economic deficit are the discarded milk from antibiotic treated cows, which cannot be put into the market, cow treatment services and drugs costs (Blosser, 1979). Moreover, subclinical mastitis infected animals are at risk for pathogen spreading within and between herds (Persson *et al.*, 2011).

1.2.2.2. *S. pyogenes* Encoded Genes in *S. dysgalactiae* subsp. *dysgalactiae*

Recently, *S. dysgalactiae* subsp. *dysgalactiae* strains carrying *S. pyogenes* virulence factors, such as superantigens, DNases, as well as antibiotic resistance genes, have been found (Rato *et al*., 2010). Some of the reported genes were phage and transposon-associated which was suggested to increase the virulence potential of *S. dysgalactiae* subsp. *dysgalactiae* since some of these genes are involved in *S. pyogenes* pathogenesis in humans (Rato *et al*., 2011). Moreover *S. dysgalactiae* subsp. *dysgalactiae* has been found in blood cultures from a human cellulitis case, following an index finger puncture from a fish dorsal fin, and in a human infective endocarditis case (Jordal *el al*., 2015; Koh *et al*., 2009). Another human case in which this species was isolated from purulent

exudate obtained from the knee of a patient after total knee arthroplasty was also reported (Park *et al*. 2012).The abovementioned publications and reports point towards the possibility of *S. dysgalactiae* subsp. *dysgalactiae*, an exclusively animal pathogen, being a potential zoonotic pathogen.

1.3. *Streptococcus dysgalactiae* subsp. *dysgalactiae* Infectious Potential

1.3.1. Human Normal and Tumoral Respiratory Cells for the study of *Streptococcus* Pathogenesis

Since many streptococcal species can colonize the respiratory tract such as the throat of humans and cause severe diseases, it is important to study the *in vitro* infectious potential on multiple types of human cell lines from this tract.
The respiratory tract is covered by continuous epithelial tissue and can be divided into three zones; the upper respiratory tract with the oral and nasal cavities, the lower respiratory tract with the larynx, trachea and bronchi, and the distal respiratory tract with the respiratory bronchioles and alveoli (BéruBé *et al*., 2009).

In vitro normal primary cell lines are usually used in order to replicate *in vivo* cell physiology due to their direct isolation from the organism (BéruBé *et al*., 2010). Once isolated, they can undergo a limited number of cell divisions before senescence while the original *in vivo* genetic background remains the same (Masters, 2000). Contrary to primary cells, tumoral cells are continuous and have the advantage of being infinitely maintained through successive cell divisions (Masters, 2000). The cost of this characteristic is the possibility of genetic background changes, which can over time lead to changes in the original cell phenotype and genotype (Masters, 2000).

Bacterial pathogenesis using *Streptococcus* species have already been systematically studied using both types of cell lines, such as normal primary bronchial/tracheal epithelial cells and the pharyngeal carcinoma epithelial cells Detroit 562 (ATCC® CCL138™) with good results, making them good *in vitro* models for

streptococcal infection and pathogenesis studies (Broudy *et al*., 2001; Broudy *et al*., 2002; Mushtaq *et al*., 2011; Okahashi *et al*., 2014; Ryan *et al*., 2001).

1.3.2. Zebrafish as a Model for *Streptococcus* Pathogenesis

Danio rerio, commonly termed zebrafish, is an animal model for the human study of embryogenesis, organ development, developmental diseases and microbe infection (Miller and Neely, 2004). Adult zebrafish have well-developed innate and adaptive immune systems resembling the human since the latter system evolved prior to the evolutionary divergence between fishes and other vertebrates (Meeker *et al.*, 2008; Saralahti *et al*., 2015). Both have T-cells, B-cells, antigen presenting cells and phagocytic cells (Saralahti *et al*., 2015). Furthermore, some granulocytes in zebrafish have behaviour similar to neutrophils in humans, when they migrate from the blood to the infected tissues during an inflammatory infection (Miller and Neely, 2004).

Other immune system components such as immunoglobulins (IgD, IgM and IgZ), cytokines and complement, and even gene homologs to mammalian genes encoding cytokines and MHC complex molecules, are present (Lewis *et al*., 2014; Saralahti *et al*., 2015).

In addition to its immune system, zebrafish has a number of advantages in regards to other animal models. These lie on its inexpensiveness compared with mammals as models, easy maintenance and small work space need due to its small size, rapid organ development, and easy breeding (Saralahti *et al*., 2015). Its small size also gives access to the disease progression in the whole animal after transverse section during histological analysis (Saralahti *et al*., 2015).

Many streptococcal species such *as Streptococcus iniae*, *Streptococcus agalactiae* and even *S. pyogenes* have been studied using this model for human disease (Saralahti *et al*., 2015).
Streptococcus iniae, a natural fish pathogen responsible for high mortality in aquaculture, has the ability to cause, in fish, localized skin infections similar to *S.*

pyogenes in humans, and multi-organ systemic infections similar to *S. agalactiae* and *S. pneumonia* also in humans (Meeker *et al.*, 2008; Saralahti *et al*., 2015). *S. iniae* is also capable of causing cellulitis in humans, similar to an *S. pyogenes* infection (Saralahti *et al*., 2015).

The pathogenesis of the major human pathogens *S. pyogenes* and *S. pneumoniae* have been studied using zebrafish as model. The former is capable of causing disease in zebrafish similar to human necrotizing fasciitis (Saralahti *et al*., 2015). In the latter case it has been demonstrated that zebrafish can immunological respond to *S. pneumonia* infection and eradicate invading bacteria which has been helpful particularly for the innate immune system study (Saralahti *et al*., 2014; Saralahti *et al*., 2015).

Together, these characteristics accentuate the importance of this animal model in the study of human disease by streptococcal species, thus helping in the study of the zoonotic potential of animal pathogens such as the potential emerging zoonotic pathogen *S. dysgalactiae* subsp. *dysgalactiae* as suggested (Jordal *el al*., 2015; Koh *et al*. 2009, Rato *et al*., 2010).

1.4. Thesis Objectives

S. dysgalactiae subsp. *dysgalactiae* has been considered as an exclusively animal pathogen. However, recently there were found *S. pyogenes* virulence genes of phage origin encoded in the *S. dysgalactiae* subsp. *dysgalactiae* genome (Rato *et al*., 2011) and it was pointed out that this subspecies should not be disregarded as a human pathogen and suggested to be an emerging zoonotic pathogen. Lacasta *et al*. (2008) and Ryan *et al*. (1991) suggested that the animal digestive tract might be a pathway for *S. dysgalactiae* subsp. *dysgalactiae* transmission and infection which highlights the possibility of animal to human transmission via infected cow milk since bovine mastitis is a disease also caused by this bacteria.

Identification of *S. dysgalactiae* subsp. *dysgalactiae* associated with human infections such as cellulitis, endocarditis and joint infection have been reported. These cases seem

to be rare and the role of this species in human pathogenesis remains unclear, which motivates further investigation in *S. dysgalactiae* subsp. *dysgalactiae* to assess if zoonotic potential exists.

It is not evident if carriage of *S. pyogenes* phage virulence genes is shared by different *S. dysgalactiae* subsp. *dysgalactiae* strains suggesting a species-specific feature. One of the goals of this thesis is to detect the presence and expression of *S. pyogenes* virulence determinants, among contemporary *S. dysgalactiae* subsp. *dysgalactiae* isolates obtained from milk samples collected from dairy herds between 2011-2013 in Portugal and compare the virulence gene patterns with the ones previously known of a collection of *S. dysgalactiae* subsp. *dysgalactiae*, isolated in 2002-2003 and previously characterized by Marcia *et al*. (2010). Another goal of this thesis is to evaluate the infection potential of *S. dysgalactiae* subsp. *dysgalactiae* isolates (selected based on virulence gene profiling), *in vitro*, using human respiratory cell lines and *in vivo*, using the zebrafish as an animal model for the study of streptococcal infections in humans.

2. Material and Methods

2.1. Bacterial isolates identification

The isolates used in this study belong to a group of 29 alpha-haemolytic Group C *Streptococcus* (GCS) *S. dysgalactiae* subsp. *dysgalactiae* (SDSD) strains isolated from milk samples of bovines from dairy herds in Portugal between 2011 and 2013. The strains were provided by Ricardo Bexiga (Faculty of Veterinary Medicine, University of Lisbon).

The presumptive identification of the isolates was performed by traditional phenotypic tests based on colony morphology, type of haemolysis in Columbia Blood Agar Base (Oxoid Ltd, Basingstoke, England) supplemented with 5% sheep blood (Probiológica, Lisbon, Portugal) (BAP), Lancefield group identification with SLIDEX Strepto Plus (Biomérieux, Marcy-l'Étoile, France) and species and subspecies identified as *S. dysgalactiae* subsp. *dysgalactiae* by PCR amplification and sequencing using generic primers for gram-positive bacteria of the 16S rRNA (forward: AGAGTTTGATCCTGGCTC; reverse: GGTTACCTTGTTACGACTT) (Takahashi *et al*., 1997). Automatic sequencing of the 1.2 kb PCR amplicon was performed on both DNA strands (STAB Vida, Lisbon, Portugal).

The DNA sequences were analyzed using the CLC Genomics Workbench 7.0.4 alignment program editor (QIAGEN, Venlo, Netherlands) and then compared with sequences from the National Center for Biotechnology Information (NCBI) database using the Basic Local Alignment Search Tool (BLAST) (www.ncbi.nlm.nih.gov/BLAST). The species and subspecies identification was carried out by Cynthia Alves Barroco (Ph.D. Student, Dept. Life Sciences. UCIBIO, Faculty of Science and Technology, NOVA University of Lisbon).

In this thesis, the same methods described above were performed to facilitate familiarization to these laboratory techniques and to obtain duplicate cultures for long-

term storage at -80ºC in Todd-Hewitt liquid medium (Oxoid Ltd, Basingstoke, England) and 30% of glycerol (VWR, Pennsylvania, USA).

2.2. DNA extraction for PCR amplification

DNA was extracted from each isolate based on a boiling method (Klugman *et al.*, 1998) in which the isolates were initially grown in BAP with one pure colony being retrieved and streaked in Todd-Hewitt (Oxoid Ltd, Basingstoke, England) agar (Liofilchem S.R.L., Roseto degli Abruzzi, Italy) supplemented with 1% (w/v) yeast extract (Oxoid Ltd, Basingstoke, England) (THA). Colonies on THA were ressuspended in 10 mM Tris-HCl pH 8.0 buffer and boiled to 100°C for 10 min. After the boiling step each sample was put in ice, to further facilitate cell lysis by thermal shock, and centrifuged at 13000 rpm for 10 min. Supernatants containing DNA were retrieved and stored at -20°C until needed. DNA concentration (at A260nm) and purity (A260nm/280nm and A260nm/A230nm) was assessed using NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific Inc, Waltham, United States of America).

Measurements of DNA samples at A260 were converted to concentration using the Beer-Lambert equation $A=\varepsilon.c.l$ (Sambrook J and Russel D, 2001) where A is the absorbance at 260 nm (A260 value); ε is the standard molar extinction coefficient for dsDNA (0.020 μg/mL in a 1 cm cuvette); c is the concentration and l is the 1 cm light pathlenght.

The ratios of A260nm/280nm and A260nm/A230nm were used to assess the purity of DNA. A value of 1.8 of the ratio A260nm/280nm and a value range of 1.8-2.2 of the ratio A260nm/230nm were considered to infer the purity of the DNA samples. When the values obtained for these ratios were superior or inferior to the expected range, new DNA extractions were conducted.

To assess correct PCR procedure, 16S rRNA was amplified as internal control, for each DNA sample, and DNA integrity was evaluated in a 1% (w/v) agarose gel electrophoresis prepared in 1X Tris-Acetate-EDTA buffer (TAE).

2.3. RNA extraction and cDNA synthesis for reverse transcription and PCR (*RT-PCR*)

All isolates were initially grown in BAP with pure colonies being retrieved and ressuspended in Todd-Hewitt broth supplemented with 1% (w/v) yeast extract (THB) and cultured at 37ºC overnight. After approximately 17 hours of incubation each bacterial culture isolate was diluted to 0.05 optical density at 600 nm (OD_{600}) in THB and incubated at 37ºC until 0.5-0.6 OD_{600}. The cells were centrifuged (at 9000 rpm for 10 minutes at room temperature) and the pellet was used for RNA extraction using the NucleoSpin RNA kit (Macherey-Nagel GmbH & Co. KG, Düren, Germany) accordingly with the manufacturer instructions. RNA concentration (A260nm) and purity (A260nm/280nm and A260nm/A230nm) was assessed using NanoDrop 1000 Spectrophotometer.

Measurements of RNA samples at A260 were converted to concentration using the Beer-Lambert equation $A=\varepsilon.c.l$ where A is the A260 value; ε is the standard molar extinction coefficient for ssRNA (0.025 μg/mL in a 1cm cuvette); c is the concentration and l is the 1 cm light pathlenght.

The ratios of A260nm/280nm and A260nm/A230nm were used to assess the purity of RNA. A value of 2.0 of the ratio A260nm/280nm and a value range of 1.8-2.2 of the ratio A260nm/230nm were considered to infer the purity of the RNA samples.

If the values obtained for these ratios were superior or inferior to the expected range, new RNA extractions would be conducted.

cDNA was generated by reverse transcription of the extracted RNA with the NZY First-Strand cDNA Synthesis Kit (NZYTech, Lisbon, Portugal) and the resulting cDNA were used as template for PCR. To assess correct RT-PCR procedure, 16S rRNA was amplified as internal control, for each cDNA sample, and integrity was evaluated in a 1% (w/v) agarose gel electrophoresis prepared in 1X TAE

2.4. Virulence genes PCR screening and expression

The following genetic determinants of group A *Streptococcus pyogenes* (GAS): *speB*, *speC*, *speF*, *speH*, *speK*, *speL*, *speM*, *smeZ*, *spd1* and *sdn* encoding a cysteine protease, 7 superantigens, and 2 extracellular nucleases were screened in all the 29 *S. dysgalactiae* subsp. *dysgalactiae* isolates by PCR. Two *Tn1207.3/Φ10394.4* sequences, a right junction (RJ) and a left junction (LJ) between this family of chimeric elements and its chromosomal insertion site, were also screened by PCR to infer the presence of either *Tn1207.3* transposon or *Φ10394.4* phage, depending on the size of the LJ amplicon (453 bp for the transposon or 6807 bp for the phage).

Primer sequences and amplicon expected sizes are listed in Table 2. For each 25 μL PCR reaction mixture were added 1 μL of bacterial DNA, 1X reaction buffer for NZYTaq DNA polymerase, 2.5 mM $MgCl_2$, 0.4 mM dNTPs NZYMix, 1U NZYTaq DNA polymerase (NZYTech, Lisbon, Portugal) and 1 μM of each primer (Thermo Fisher Scientific, Waltham, United States of America). PCR conditions for amplification of all the genetic determinants are listed in Table 3.
Negative results were confirmed after at least two repetitive results.

Table 2. Primer sequences, amplicon expected size and PCR control strains for the *Streptococcus pyogenes* virulence factors screened in *Streptococcus dysgalactiae* subsp. *dysgalactiae* isolates under study.

Virulence factor	**Location**	**Primer sequence (5'-3')**	**Amplicon expected size (bp)**	**Ref.**	**Species/ Control Strain**
Cysteine protease (*speB*)	Chromosome	for.: TTCTAGGATACTCTACCAGC rev.: ATTTGAGCAGTTGCAGTAGC	300	Jasir *et al.* (2001)	*S. pyogenes* S13
Streptococcal pyrogenic exotoxin C (*speC*)	Phage	for.: GCAGGGTAAATTTTTCAACGACACACA rev.: TGTGCCAATTTCGATTCTGCCGC	407	Rato (2011)	S. *dysgalactiae* subsp. *dysgalactiae* VSD13
Streptococcal pyrogenic exotoxin F (*speF*)	Chromosome	for.: TACTTGGATCAAGACG rev.: GTAATTAATGGTGTAGCC	782	Schmitz *et al.* (2003)	*S. pyogenes* S13
Streptococcal pyrogenic exotoxin H (*speH*)	Phage	for.: TCTATCTGCACAAGAGGTTTGTGAATGTCC rev.: GCATGCTATTAAAGTCTCCATTGCCAAAA	338	Pires (2011)	*S. pyogenes* GAS 1002
Streptococcal pyrogenic exotoxin K (*speK*)	Phage	for.: TACAAATGATGTTAGAAATCCAAGGAACATATATGCT rev.: CAAAGTGACTTACTTTACTCATATCAATCGTTTC	656	Rato (2011)	S. *dysgalactiae* subsp. *dysgalactiae* VSD13
Streptococcal pyrogenic exotoxin L (*speL*)	Phage	for.: CTGTTAGGATGGTTTCTGCGGAAGAG rev.: AGCACCTTCCTCTTTCTCGCCT	605	Rato (2011)	S. *dysgalactiae* subsp. *dysgalactiae* VSD13
Streptococcal pyrogenic exotoxin M (*speM*)	Phage	for.: CCAATATGAAGATAACAAAGAAAATTGGCA rev.: CAAAGTGACTTACTTTACTCATATCAATCG	600	Rato (2011)	S. *dysgalactiae* subsp. *dysgalactiae* VSD13
Streptococcal mitogenic exotoxin Z (*smeZ*)	Chromosome	for.: CAGATATAGTAATTGATTTTA rev.: AGCTAGAACCAGAAGAATAT	399	Darenberg *et al.* (2007)	*S. pyogenes* GAP58
Streptodornase (*sdn*)	Phage	for.: ACCCCATCGGAAGATAAAGC rev.: AACGTTCAACAGGCGCTTAC	489	Matsumoto *et al.* (2008)	S. *dysgalactiae* subsp. *dysgalactiae* VSD7
DNase1 (*spd1*)	Phage	for.: CCCTTCAGGATTGCTGTCAT	400	Green *et al.*	S. *dysgalactiae*

		rev.: ACTGTTGACGCAGCTAGGG		(2005)	subsp. *dysgalactiae* VSD13
Tn1207.3/Φ10394.4 LJ	Chimeric element	for.: TCTTCGCCGCATAAACCCTATC rev.: CCTTTGACCAATGAAGTGACCTTT	453/6807	Figueiredo *et al.* (2006)	S. *dysgalactiae* subsp. *dysgalactiae* VSD13
Tn1207.3/Φ10394.4 RJ	Chimeric element	for.: CGAGGAGTTAGTATGGAAAC rev.: CCCATAATAGGCAACTGGTCTCCAGC	473	Figueiredo *et al.* (2006)	*S. pyogenes* CSO5012

LJ – Left junction; RJ – Right junction.

Table 3. PCR amplification conditions for the *Streptococcus pyogenes* virulence factors screened in *Streptococcus dysgalactiae* subsp. *dysgalactiae* isolates under study.

	Initial Denaturation		Denaturation		Annealing		Extension		Final extension		No. of cycles	Ref.
Gene	T	t	T	t	T	t	T	t	T	t		
speB	95º	5’	94ºC	1'	58ºC	2'	72ºC	1'	72ºC	7’	35	Jasir *et al.* (2001)
speC	95º	5’	94ºC	30"	60ºC	90"	72ºC	90"	72ºC	7'	30	Rato (2011)
speF	95º	5’	94ºC	1'	58ºC	2'	72ºC	1'	72ºC	7’	35	Schmitz *et al.* (2003)
speH	95º	5’	94ºC	30"	59ºC	90"	72ºC	90"	72ºC	7’	30	Pires (2011)
speK	95º	5’	94ºC	30’’	57ºC	30’’	72ºC	1’	72ºC	7’	30	Rato (2011)
speL	95º	5’	94ºC	30’’	60ºC	30’’	72ºC	45’’	72ºC	7’	35	Rato (2011)
speM	95º	5’	94ºC	30’’	60ºC	30’’	72ºC	45’’	72ºC	7’	35	Rato (2011)
smeZ	96ºC	5’	96ºC	50’’	49ºC	65’’	72ºC	70’’	72ºC	5’	30	Darenberg *et al.* (2007)
sdn	95ºC	5’	95ºC	30"	65ºC	30"	72ºC	45’’	72ºC	7’	32	Matsumoto *et al.* (2008).
spd1	95º	5’	95ºC	30"	60ºC	30"	72ºC	45’’	72ºC	7’	32	Green *et al.* (2005)
Tn1207.3/Φ10394.4 (LJ and RJ)	94ºC	5’	94ºC	30’’	60ºC	40’’	72ºC	2’	72ºC	5’	35	Figueiredo *et al.* (2006)

T – Temperature; t – Time; LJ – Left Junction; RJ – Right Junction

2.5. DNase semi-quantitative assay

The assessment of extracellular DNase production was performed based on Sumby *et al.* (2005). Isolates were initially grown as already described in subsection 2.3. After approximately 17 hours of incubation each bacterial culture isolate was diluted in Todd-Hewitt broth supplemented with 0.5% (w/v) yeast extract and incubated at 37ºC until stationary phase. Bacterial cultures were then centrifuged at 3000 rpm for 10 minutes and supernatants filtered (0.2µm).

For each 50 µL of reaction mixture were added 10 µL supernatant, 1 µg dsDNA (known PCR generated amplicons), 1X SuRE/Cut Buffer M (Sigma-Aldrich Co. LLC, St. Louis, United States of America) and sterile Milli-Q ultrapure water (Millipore Corporation). Each reaction mixture was then incubated at 37ºC for 1h. For the negative control, 10 µL sterile Milli-Q water was used instead of culture filtered supernatant. After incubation, 10 µL from each mixture was analysed in a 1% (w/v) agarose gel electrophoresis (prepared in 1X TAE buffer).

2.6. Bacterial growth curve analysis

Bacterial growth was assessed by colony forming units per millilitre (CFU/mL) method and the optical density at 600 nm (OD_{600}). Isolates were initially grown as described in subsection 2.3. After approximately 17 hours of incubation each bacterial culture isolate was diluted in new THB medium and cultured at 37ºC without agitation for 24 hours. Immediately before incubation, a sample of the bacterial culture was used for OD_{600} measurement, and serial diluted and plated in THA, to determine the initial CFU/mL. After this, samples of the bacterial cultures were collected every hour for the following 8 hours and a last sample after 24hours of incubation to evaluate the OD_{600} and CFU/ml values. THA plates with the diluted cultures were incubated at 37ºC for approximately 17 hours and the CFU/ml values were determined.

2.7. *In vitro* human cell line infection assay

For the *in vitro* infection analysis three SDSD isolates were chosen based on their virulence gene profiles and one *S. pyogenes* invasive strain isolated from the blood of a human septicaemia patient (Table 4).

Table 4. Streptococcal isolates chosen for the *in vitro* infection assays based on virulence genotypes.

Species	Strain code	GAS virulence genes detected	Clinical origin	Ref.
Streptococcus dysgalactiae subsp. *dysgalactiae*	VSD21	none	Sub-clinical mastitis	This study
	VSD23	*sdn*	Sub-clinical mastitis	
	VSD24	*speC, speK, spd1*	Clinical mastitis	
Streptococcus pyogenes	GAP58	*speA, speB, speF, speJ, smeZ*	Invasive infection	Pires (2011)

Isolates were initially grown as described in subsection 2.3. After approximately 17 hours of incubation each bacterial culture isolate was diluted to 0.05 OD_{600} in new THB medium and cultured at 37°C until mid-exponential phase and washed three times with the same sterile culture medium, and finally ressuspended and diluted in antibiotic-free Dulbecco's Modified Eagle Medium (DMEM) (Thermo Fisher Scientific Inc, Waltham, United States of America) supplemented with 10% (v/v) fetal bovine serum (FBS) (Thermo Fisher Scientific Inc, Waltham, United States of America).

The human cell lines used in this study were the pharyngeal carcinoma epithelial cells Detroit 562 (ATCC® CCL138™) and the normal Primary Bronchial/Tracheal Epithelial Cells (BTEC) (ATCC® PCS300010™). Confluent monolayers (concentration of $3x10^5$ cells/mL) for *in vitro* infection assays were prepared by subculture of cell monolayers into 96-well cell culture plates (Sigma-Aldrich Co. LLC, St. Louis, United States of America) and incubation overnight at 37°C in a CO_2 5% atmosphere. Human cell line

cultures were performed by Catarina Roma-Rodrigues (Post-Doc at Dept. Life Sciences, UCIBIO, Faculty of Science and Technology, NOVA University of Lisbon).
Human cells were then washed three times with 1X phosphate buffer saline (PBS) and bacterial suspensions added on top of the human cell monolayer (multiplicity of infection of 1:100). Bacterial suspensions were simultaneously plated on THA, to confirm the number of bacteria added to each well through the assessment of CFU/mL. The 96-well cell culture plate was then incubated for 2 hours at 37°C in a 5% (v/v) CO_2 atmosphere.

After the 2 h period the supernatant in each well was removed by washing 3 times in 1X PBS (to remove all extracellular non-adherent bacteria) and used for assessing the number of CFU/mL. Cell monolayers were then detached and collected from each well through the addition of TrypLE Express Enzyme (Thermo Fisher Scientific, Waltham, United States of America) and finally lysed in 0.1% (v/v) Triton X-100 (Sigma-Aldrich Co. LLC, St. Louis, United States of America) to recover intracellular and extracellular adhered bacteria which were serial diluted and plated on THA to determine CFU/mL, and percentage of adherent and intracellular bacteria by relation with the initial infection CFU count (% Adhered and Internalized Streptococci).

Infection analysis was performed using the statistical analysis software SigmaPlot 12.0 (Systat Software Inc, San Jose, United States of America) using Student's t-test method

2.8. *In vivo* animal infection assay

For the *in vivo* infection analysis of the *S. dysgalactiae* subsp. *dysgalactiae* isolate VSD24 (*speC*, *speK*, *spd1*) was selected. The *in vivo* model used in the study was *Danio rerio* (zebrafish) obtained from national suppliers (Aquaplante, Lisbon, Portugal) and housed at the FCT/UNL fish facilities following essentially the acclimation and experiment conditions described for zebrafish (Diniz *et al.*, 2015).

All the experiments followed the international welfare regulations and were previously approved by "Direcção Geral de Veterinária". Sterile Tryptic Soy Broth (Becton, Dickinson Company, East Rutherford, United States of America) (TSB) medium was used as negative control and *S. pyogenes* invasive strain GAP58 (*speA, speB, speF, speJ, smeZ*) was used as positive control.

Isolates were initially grown as described in subsection 2.3. After approximately 17 hours of incubation each bacterial culture isolate was diluted in new THB medium and cultured at 37ºC until mid-exponential phase and washed, and ressuspended in TSB culture medium. Three groups of zebrafish were injected intraperitoneally with 10 µL of culture medium containing $1x10^7$ bacterial cells (SDSD VSD24 or *S. pyogenes* GAP58) and 10 µL of sterile culture medium (Control group), using a NanoFil 10 µL syringe (World Precision Instruments, Sarasota, United States of America). From the total 31 injected zebrafish under study, 15 zebrafish were injected with sterile TSB, 9 with GAP58 strain and 7 with the selected SDSD isolate.
Injection of zebrafish was performed by Mário Diniz (Assistant Professor, Dept. Chemistry, UCIBIO, Faculty of Science and Technology, NOVA University of Lisbon).

The three zebrafish groups were then separately maintained in aquaria at 28ºC, using a heated water bath circulator (Haake D1, Haake Messtecknik GmbH Co., Karlsruhe, Germany), without being fed during the 15 days of assay, or until death in which they were analysed. After the 15 days, remaining alive fish were euthanized and dissected for the analysis of the intraperitoneal region (fish viscera) and caudal peduncle (fish muscle). Both were homogeneised in 1X PBS, with the muscle homogenization being further centrifuged at 2000 rpm for 10 minutes, and both plated on BAP medium supplemented with 10 µg/mL tetracycline in order to select both isolates (VSD24 and GAP58) under study. Susceptibility to tetracycline was previously determined by Cinthia Alves Barroco (Ph.D. student).

Analysis of dead fish was done in order to determine if the causal agent of death was the respective injected bacterial isolate. Control zebrafish were used in order to assess the

stress caused by the injection. The presumptive identification of the *S. dysgalactiae*, subsp. *dysgalactiae* and *S. pyogenes* isolates was performed as described in subsection 2.1.

Infection analysis was performed using the statistical analysis software SigmaPlot 12.0 through Kaplan-Meyer's Survival Analysis in which the Log-Rank method was performed for survival curves, followed by the Holm-Sidak method for multiple comparison procedures.

3. Results and Discussion

3.1. Virulence factors screening by PCR and expression by RT-PCR

- <u>Overall virulence gene distribution</u>

Of the total 29 *Streptococcus dysgalactiae* subsp. *dysgalactiae* (SDSD) bovine isolates screened by PCR, approximately 66% (19 isolates) carry at least one of the six following group A *Streptococcus pyogenes* (GAS) virulence genes: *speC*, *speK*, *speL*, *speM*, *spd1* and/or *sdn*, all encoded by bacteriophages. The remaining 10 isolates (VSD20, VSD21, VSD22, VSD30, VSD31, VSD34, VSD38, VSD39, VSD45 and VSD46) do not carry any of the 10 virulence genes that were screened, including the abovementioned and phage-associated *speH*, and chromosome-associated *speB*, *speF*, and *smeZ* genes. None of the 29 isolates carry the *speF* and *speH* pyrogenic exotoxin genes, *speB*, and the *smeZ* mitogenic exotoxin gene. These results are shown in Table 5.

Table 5. Overview of the 19 *Streptococcus dysgalactiae* subsp. *dysgalactiae* isolates with at least one *S. pyogenes* virulence gene detected by PCR.

***Streptococcus dysgalactiae* subsp. *dysgalactiae* strain**	**Farm Code**	***S. pyogenes* virulence genes[1] detected**
VSD23	V	*sdn*
VSD24	S	*speC*, *speK*, *spd1*
VSD25	N	*speM*, *sdn*
VSD26	N	*speK*, *speL*
VSD27	M	*speL*, *speM*
VSD28	X	*speM*, *sdn*
VSD29	M	*speM*
VSD32	O	*speK*, *sdn*
VSD33	O	*speK*, *sdn*
VSD35	N	*speM*, *sdn*
VSD36	M	*speL*, *speM*
VSD37	O	*speK*, *sdn*
VSD40	R	*speM*, *sdn*
VSD41	M	*speL*, *speM*
VSD42	M	*speL*, *speM*
VSD43	M	*speK*, *sdn*
VSD44	S	*speC*, *speK*, *spd1*
VSD47	M	*speC*, *speK*, *spd1*
VSD48	M	*speC*, *speK*, *spd1*

[1]Genes *speC*, *speK*, *speL*, *speM*, *spd1* and *sdn* are encoded by *S. pyogenes* phages.

The *sdn* gene was detected in 31% of the isolates (9 isolates), *speK* in 31% (9 isolates), *speM* in 31% (9 isolates), *speL* in 17% (5 isolates), and *speC* and *spd1* were detected simultaneously in 14% of the isolates (4 isolates).

Transcriptional analysis showed that the virulence genes *speC, speK* and *spd1* were transcribed in all SDSD isolates in which these genes were detected by PCR screening. The gene *speL* was transcribed in all isolates except in VSD41 and the *sdn* gene was also transcribed in all isolates except in VSD25. The *speM* gene was not transcribed in two isolates (VSD29 and VSD41). These results are shown in Table 6.

Table 6. Transcription results obtained by RT-PCR of virulence genes detected in 19 SDSD isolates (out of 29 isolates) with at least one *S. pyogenes* phage virulence gene.

***Streptococcus dysgalactiae* subsp. *dysgalactiae* strain**	**Transcription of *S. pyogenes* virulence genes**
VSD23	*sdn* $^{+}$
VSD24	*speC* $^{+}$, *speK* $^{+}$, *spd1* $^{+}$
VSD25	*speM* $^{+}$, *sdn* $^{-}$
VSD26	*speK* $^{+}$, *speL* $^{+}$
VSD27	*speL* $^{+}$, *speM* $^{+}$
VSD28	*speM* $^{+}$, *sdn* $^{+}$
VSD29	*speM* $^{-}$
VSD32	*speK* $^{+}$, *sdn* $^{+}$
VSD33	*speK* $^{+}$, *sdn* $^{+}$
VSD35	*speM* $^{+}$, *sdn* $^{+}$
VSD36	*speL* $^{+}$, *speM* $^{+}$
VSD37	*speK* $^{+}$, *sdn* $^{+}$
VSD40	*speM* $^{+}$, *sdn* $^{+}$
VSD41	*speL* $^{-}$, *speM* $^{-}$
VSD42	*speL* $^{+}$, *speM* $^{+}$
VSD43	*speK* $^{+}$, *sdn* $^{+}$
VSD44	*speC* $^{+}$, *speK* $^{+}$, *spd1* $^{+}$
VSD47	*speC* $^{+}$, *speK* $^{+}$, *spd1* $^{+}$
VSD48	*speC* $^{+}$, *speK* $^{+}$, *spd1* $^{+}$

+ gene transcribed; - gene not transcribed

As described by Broudy *et al*. (2002), Beres and Musser (2007), Korczynska *et al*. (2012) and other authors, the *speC* and *spd1* genes are always found in linkage in the same prophage genome (such as the phages *ϕ370.1*, *ϕ10270.1*, *ϕ10750.1* and *ϕ10394.5*, for example), and in fact these were also found in linkage in isolates VSD24, VSD44, VSD47 and VSD48 (see Table 5). Furthermore, in addition to these genes, *speK* was

also shown to be present on those abovementioned four strains. As shown by several authors, this genotype profile is characteristic of *S. pyogenes* strains, such as MGAS6180, MGAS10394 and MGAS10270, carrying prophages with the *speC-spd1* and *speK* genes (Banks *et al*., 2004; Green *et al*., 2005b; Beres and Musser, 2007).

Linkage between *speL-speM* genes was also found in isolates VSD27, VSD36, VSD41 and VSD42. This might be due to the *ϕ8232.3* phage in which these genes are also in linkage (Smoot *et al.*, 2002; Beres and Musser, 2007). Interestingly *speM* was found in isolates VSD25, VSD28, VSD29, VSD35 and VSD40 independently of *speL*, and *speL* was found in VSD26 independently of *speM*, which might suggest the existence of a variant of the *ϕ8232.3* phage with different genomic organization. Nevertheless, the inability to find gene linkage in these 6 isolates could be due to primer design.

- <u>Virulence gene distribution by farm</u>

As shown in Table 7, out of the total 11 farms where SDSD isolates were retrieved, farm coded as P, Q, T and U were the only farms where no SDSD strains were found carrying *S. pyogenes* virulence genes.

Farm M contributed with the greatest number of SDSD isolates (n=9) with five different genotypes. VSD31 was the only isolate from this farm without *S. pyogenes* virulence genes detected. Linkage between *speL-speM* genes was only found in four isolates from this farm. As for VSD25, VSD28, VSD29, VSD35 and VSD40, in which *speM* was found to not be in linkage with *speL*, these were dispersed among this farm and farms N, R and X. In addition, all the isolates from other farms carrying the *speM* gene also carried the *sdn* gene. The latter gene was detected in 6 out of 7 farms where at least one *S. pyogenes* virulence gene was found.

In farm M there were also isolated VSD47 and VSD48 carrying the *speC* and *spd1* genes, also probably in linkage, as discussed above. Farm S was the other farm where the same genotype was found. (VSD24 and VSD44). *speC-spd1* as reported by Green *et*

al. (2005), was found in a larger proportion (84%) of *S. pyogenes* M28 strains, and of other M types (Beres and Musser 2007). Our data suggest that these two genes are not so well disseminated among bovine *S. dysgalactiae* subsp. *dysgalactiae* strains. This is interesting since it was already demonstrated by Broudy *et al*. (2002) that *S. pyogenes* prophage induction, harbouring *speC-spd1*, occurs when the bacteria interacts with human pharyngeal cell cultures. It would be interesting to see if the same occurs with these SDSD strains, and if not (in the presence of a defective phage for example), it could explain the low frequency of this genotype in the isolates and low dissemination throughout the farms. On the other hand, all these isolates were isolated from cases of bovine mastitis, which implies a different tissue than pharyngeal cells, particularly human.

Table 7. Overview of the farms and virulence gene distribution of the *Streptococcus dysgalactiae* subsp. *dysgalactiae* isolates.

Farm Code	Virulence genes	***Streptococcus dysgalactiae* subsp. *dysgalactiae* isolates**
M	*speL*, *speM*	VSD27, VSD36, VSD41, VSD42
	speM	VSD29
	-	VSD31
	speK, *sdn*	VSD43
	speC, *speK*, *spd1*	VSD47, VSD48
N	*speM*, *sdn*	VSD25, VSD35
	speK, *speL*	VSD26
O	*speK*, *sdn*	VSD32, VSD33, VSD37
P	-	VSD34
Q	-	VSD21, VSD22
R	*speM*, *sdn*	VSD40
S	-	VSD20
	speC, *speK*, *spd1*	VSD24, VSD44
T	-	VSD30
U	-	VSD35, VSD38, VSD39, VSD46
V	*sdn*	VSD23
X	*speM*, *sdn*	VSD28

- **Comparative distribution of virulence factors**

As mentioned above, these results demonstrate that phage-associated virulence factors from *S. pyogenes* are present and expressed in bovine SDSD isolates collected from dairy herds between 2011 and 2013 in Portugal as it was previously reported for bovine

SDSD isolated in Portuguese herds in 2002-2003 (Rato *et al*., 2010). Interestingly, not all isolates from the collection under study transcribed these genes (see Table 6), as opposed to the previous collection were all isolates, in which they were present, expressed them.

A comparison of the distribution of these virulence determinants in previously characterized isolates collected in 2002 and 2003 and the most recent isolates, under study, is present in Table 8.

Table 8. Distribution (%) of group A *Streptococcus pyogenes* (GAS) virulence genes in *Streptococcus dysgalactiae* subsp. *dysgalactiae* collections isolates in 2002-2003 and 2011-2013.

GAS virulence factors detected in *Streptococcus dysgalactiae* subsp. *dysgalactiae* isolates	***Streptococcus dysgalactiae* subsp. *dysgalactiae* 2002-2003 collection % (n=18) [1]**	***Streptococcus dysgalactiae* subsp. *dysgalactiae* 2011-2013 collection % (n=29) [1]**
Pyrogenic exotoxin C (*speC*)	33 (n=6)	14 (n=4)
Pyrogenic exotoxin K (*speK*)	50 (n=9)	31 (n=9)
Pyrogenic exotoxin L (*speL*)	22 (n=4)	17 (n=5)
Pyrogenic exotoxin M (*speM*)	11 (n=2)	31 (n=9)
DNase1 (*spd1*)	33 (n=6)	14 (n=4)
Streptodornase (*sdn*)	22 (n=4)	31 (n=9)
Composite transposon (*Tn1207.3/Φ10394.4*)		
Left Junction	100 (n=18)	100 (n=29)
Right Junction	0 (n=0)	0 (n=0)
At least one gene	72 (n=13)	66 (n=19)
Reference	Rato *et al.*, 2011	This study

[1]Chi-squared statistical analysis revealed that each independent virulence gene proportion was not statistically different in between collections.

In all the 29 SDSD strains isolated there was no amplification of the *Tn1207.3/Φ10394.4* chimeric element right junction (RJ) and screening of the left junction (LJ) showed PCR amplicon sizes lower than 400 bp, as opposed to the expected 453-6807 bp described for *S. pyogenes* (Figueiredo *et al.*, 2006), as seen previously in the 18 characterized *S. dysgalactiae* subsp. *dysgalactiae* isolates collected in 2002 and 2003 (Rato *et al.*, 2010).

Despite this observation, *Tn1207.3/Φ10394.4* chimeric element, inserted in the *comEC* locus, may be present in and be a characteristic of all strains of *S. dysgalactiae* subsp.

dysgalactiae of bovine origin since *comEC-Tn1207.3* left junction was present in all isolates from all farms in both collections, which are roughly 10 years apart. On the other hand the fact that the *Φ10394.4-comEC* right junction of this element was not detected in all isolates from both collections suggests that *a)* only *Tn1207.3* transposon is present or *b)* a different *Tn1207.3/Φ10394.4* related element is present. Neverthless the inability to find the RJ could be due to primer design. In regards to the LJ lower amplicon size and point *b)*, this element in *S. dysgalactiae* subsp. *dysgalactiae* species might have a different genomic organization in comparison with *S. pyogenes*.

This report of Rato *et al*. (2010) was the first and only report so far of *S. dysgalactiae* subsp. *dysgalactiae* carrying superantigens and DNases of *S. pyogenes*. Together, the results from both collections suggest that despite gene frequency variation among farms and/or period of isolation, statistical analysis revealed that each independent virulence gene proportion was not statistically significantly different (*p value* > 0.05) among collections (*speC p* = 0.1116; *speK p* = 0.1935; *speL p* = 0.6731; *speM p* = 0.1168; *spd1 p* = 0.1116; *sdn p* = 0.5115).

Together, with the observation that, these virulence factors are found in strains isolated almost 10 years apart suggest that they were not randomly found in the 2002-2003 collection, and are most probably a characteristic of *S. dysgalactiae* subsp. *dysgalactiae* strains of bovine origin. This is further emphasized by the inability of Abdelsalam *et al*. (2010) to find some of these virulence genes, such as *speB, speC, speM* and *smeZ*, in strains isolated from fish in other countries in Asia. However in another study, Chénier *et al*. (2008) tried, unsuccessfully, to identify various *S. pyogenes* superantigens (*speA, speC, speG, speH, speI, speJ, speK, speL, speM, ssa* and *smeZ*) in a *S. dysgalactiae* subsp. *dysgalactiae* strain isolated from a cow in Canada.

The presence of several streptococcal exotoxins in the *S. dysgalactiae* subsp. *dysgalactiae* isolates from both collections, namely SpeC, SpeK, SpeL and SpeM, might suggest that these strains have the potential to induce T-cell hyper-stimulation and thus lead to life-threatening systemic infections. However, the previously discussed

report from Chénier et al. (2008) did not found these genes in the cellulitis case associated with toxic shock-like syndrome.

Since *S. pyogenes* strains with these virulence genes such as MGAS8232 (*speC-spd1*, *speL-speM*), MGAS6180 (*speC-spd1*, *speK-sla*, *sdn*) and MGAS315 (*sdn*, *speK-sla*), are known to cause in humans rheumatic fever, pharyngitis and toxic-shock syndrome, respectively, it further emphases the potential for the *S. dysgalactiae* subsp. *dysgalactiae* isolates, particularly those under study, to cause similar diseases, as seen on the reported cases of cellulitis episode described by Koh *et al*. (2009) and endocarditis described by Jordal *et al.* (2015) (Smoot *et al*., 2002; Green *et al*., 2005b; Musser *et al*., 1991). In the latter report the isolate under study was found to have a multi-locus sequence typing (MLST) profile matching a *S. dysgalactiae* subsp. *dysgalactiae* strain from bovine origin.

- Horizontal genetic transfer (HGT) potential between *S. pyogenes* and *S. dysgalactiae* subsp. *dysgalactiae*

The previous observations that all *S. pyogenes* virulence genes detected in SDSD are phage-associated and that lytic cycle of these phages can be induced in *S. pyogenes*, point towards the possibility of HGT events. Throughout the years, these have already been documented between *S. pyogenes* and other streptococcal species, as observed in studies from Towers *et al*. (2004), Giovanetti *et al*. (2008) and Vojtek et al. (2008). In the former study it was hypothesized that *S. dysgalactiae* subsp. *equisimilis* acquired a superantigen from a *S. pyogenes* phage, and in the latter it was demonstrated *S. pyogenes* phage transduction to *S. dysgalactiae* subsp. *equisimilis*.
To date, genetic transfer has not been demonstrated between *S. pyogenes* and *S. dysgalactiae* subsp. *dysgalactiae*.

HGT events involving *S. pyogenes* have also been evidenced in a Broudy and Fischetii (2003) study in which a non-toxigenic *S. pyogenes* strain became toxygenic upon *S. pyogenes* toxygenic phage induction within the host. The fact that this study also proved

the occurrence of phage transduction and convert recipient bacteria in the second host into toxigenic, might explain how a predominantly bovine pathogen such as *S. dysgalactiae* subsp. *dysgalactiae* carries *S. pyogenes* phage virulence genes despite their radically different niches. The fact that in the present thesis all virulence genes found in the SDSD isolates are phage-encoded, and the lack of *S. pyogenes* chromosomal virulence genes, also point to this possibility.

3.2. Extracellular DNase expression

All the 29 *S. dysgalactiae* subsp. *dysgalactiae* isolates were found to express extracellular DNases with the ability to degrade 1 µg dsDNA in 1 hour incubation at 37°C after the addition of 10 µl culture filtered supernatant. A representation of these results is shown in Figure 4 for VSD21, VSD22, VSD23, VSD24, VSD25 and VSD26 strains.

DNase1 (Spd1) and streptodornase (Sdn) presence in 14% (n=4) and 31% (n=9) of the isolates respectively, had no correlation with the observed *in vitro* DNase activity since it appears to be independent on the presence and expression of these two genes (see Tables 5 and 6). Together these results suggest that other streptococcal extracellular nucleases must be in play, which seems plausible since most *S. pyogenes* strains have at least two DNase genes, as seen in a study from Beres and Musser (2007), such as the above studied and others such as Spd3, Spd4 and Sda. All these are encoded on mobile genetic elements, particularly prophages, as also seen in other studies (Sumby et al., 2005b; Aziz et al., 2004). These enzymes might allow *S. dysgalactiae* subsp. *dysgalactiae* to escape neuthrophil NETs and thus, evade the innate immune system response during an inflammatory infection. This is of extremely importance since these strains were the cause of bovine mastitis in which neuthrophils normally migrate from the blood to the mammary gland during the intramammary infection.

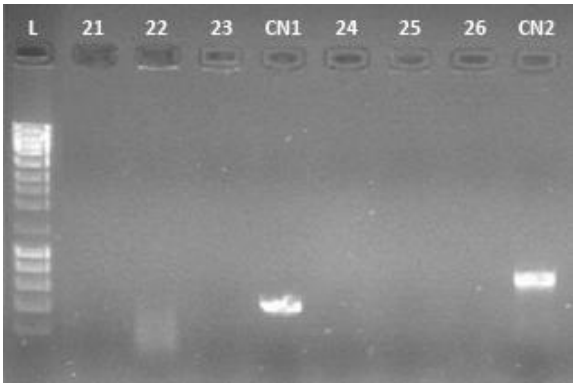

Figure 4. Representation of extracellular DNase production by selected strains. L – NZYDNA Ladder III (NZYTech, Lisbon, Portugal); CN1 – 418bp dsDNA control without bacterial supernatants; CN2 – 700bp dsDNA control without bacterial supernatants; 21 – supernatant of VSD21 with CN1 control DNA; 22 – supernatant of VSD22 with CN1 control DNA; 23 – supernatant of VSD23 with CN1 control DNA; 24 – supernant of VSD24 with CN2 control DNA; 25 – supernant of VSD25 with CN2 control DNA; 26 – supernant of VSD26 with CN2 control DNA; Results obtained by a 1% agarose gel eletrophoresis in 1X TAE; 90V for 1 hour.

3.3. Growth curves analysis

As previously mentioned (see Materials and Methods section 2.6.), the growth curves of three selected SDSD culture isolates were analysed in order to accurately determine the relation between colony forming units per millilitre (CFU/mL) and respective OD_{600}. Growth measurements in OD_{600} and CFU/mL are displayed in Figure 5. Strains VSD21 (without *S. pyogenes* virulence genes), VSD23 (*sdn*) and VSD24 (*speC*, *speK* and *spd1*) were chosen to be studied *in vitro*, and VSD24 for the *in vivo* infection assay discussed below.

S. pyogenes GAP58 strain growth curve was previously determined by colleagues.

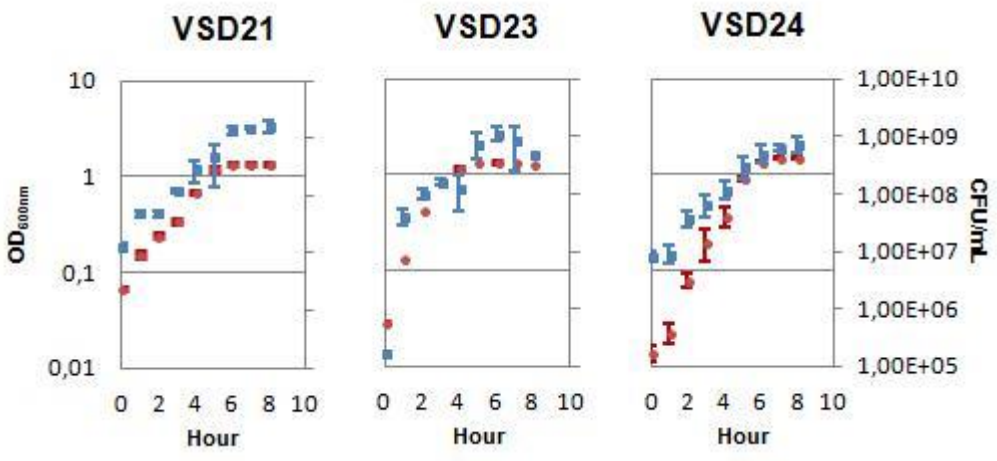

Figure 5. Growth curve analysis of the three *S. dysgalactiae* subsp. *dysgalactiae* isolates VSD21, VSD23 and VSD24. Optical density at 600 nm (OD_{600}) (red circles) and colony forming units per millilitre (CFU/mL) (blue squares) are depicted.

3.4. *In vitro* human cell line infection assay

The *in vitro* infection assay method used does not allow a discrimination between intracellular and extracellular adhered bacteria probably due to their observed ability to form biofilm, on the 96-well cell culture plates (data not shown) while the assay was being optimized. The biofilm matrix could work as a barrier protecting the bacteria from antimicrobials (Marks *et al*. 2014).

The bacterial strains selected for this assay were, as previously stated on section 2.7 of Materials and Methods, *S. dysgalactiae* subsp. *dysgalactiae* bovine isolates VSD21 (without *S. pyogenes* virulence genes detected), VSD23 (*sdn*) and VSD24 (*speC*, *speK* and *spd1*) chosen through the basis of their virulence genes profile and *S. pyogenes* GAP58 (*speA*, *speB*, *speF*, *speJ*, *smeZ*) human invasive strain (see Table 4 in section 2.7 of Material and Methods).

- **Pharyngeal carcinoma epithelial cells Detroit 562**

Infection of Detroit 562 pharyngeal carcinoma human cell line with the SDSD isolates selected showed a residual percentage of adherence and internalization (bacterial interaction) compared to the *S. pyogenes* GAP58 strain (1.24% for VSD21, 0.50% for VSD23 and 0.32% for VSD24 vs. 3.42%) (Figure 6). Statistical analysis showed that these results for all three SDSD isolates were statistically significantly different ($p \leq 0.05$) compared to GAP58 infection which indicates a difference in the infection potential of these bacteria. Moreover, the low adherence and internalization of SDSD isolates suggest a lack of interaction with this type of human cell line (Detroit 562).

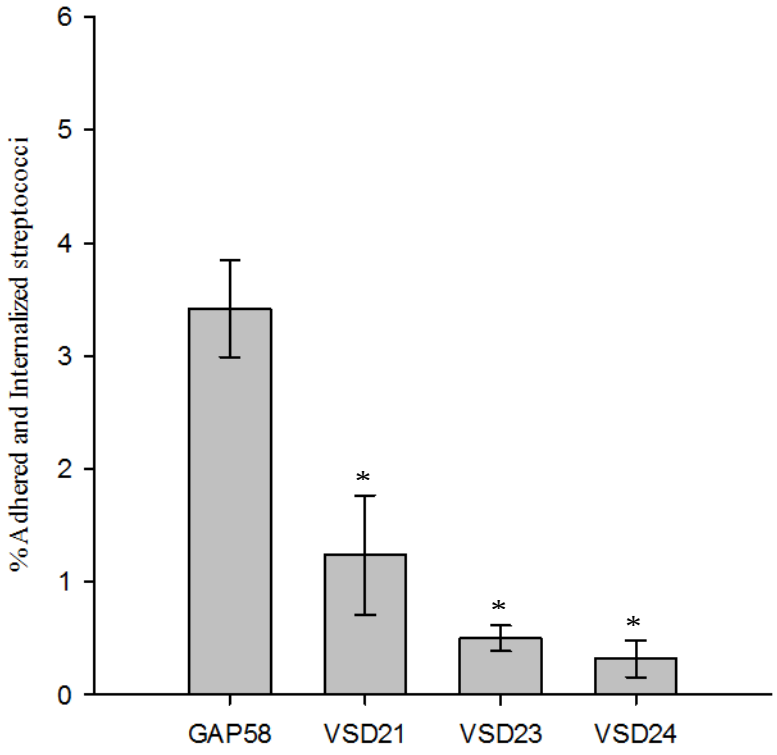

Figure 6. Percentage of adhered and internalized (interaction) streptococci in Detroit 562 human cell line. VSD21 – SDSD isolate without virulence genes detected ($p = 0.05$; 4 assays); VSD23 – SDSD isolate with one virulence gene (*sdn*) detected ($p = 0.02$; 4 assays); VSD24 – SDSD isolate with three virulence genes (*speC*, *speK*, *spd1*) detected ($p = 0.021$; 2 assays); GAP58 – *S. pyogenes* infection control invasive strain isolated from human blood. * – ($p \leq 0.05$; 3 assays). Statistical group comparison was performed using Student's t-test method.

VSD21, the SDSD isolate without any *S. pyogenes* virulence genes detected, was shown to interact more with Detroit 562 than any other contemporary isolate. From these three isolates, and despite the fact that the bacterial inoculum on the 96-well culture plate had slightly lower CFU/mL count compared to the other strains, VSD21 still managed to interact more with this human cell line as observed in the adherent and internalized CFU/mL determination. Despite this, statistical analysis showed no significant differences (*p value* > 0.05) between infection of these isolates when compared to each other (VSD23 $p = 0.232$ and VSD24 $p = 0.377$ vs. VSD21, and VSD23 $p = 0.490$ vs. VSD24).

It is apparent that the results from these specific isolates with lower interaction with Detroit 562 than the *S. pyogenes* invasive isolate, are contrary to the results of *S. dysgalactiae* subsp. *dysgalactiae* VSD5, VSD9 and VSD13 isolates from the 2002-2003 collection (see Table 11 and Figure 11 in section 6. Annex) (studied by C. Roma-Rodrigues and C. Alves-Barroco). These latter strains are shown to adhere to or internalize into Detroit 562 with no statistical significant difference compared to GAP58 control strain, dispite the higher percentage of interaction of these strains.

Together, the results from both collections suggest that bovine SDSD strains seem to have different ability to interact with Detroit 562, possibly caused by different virulence genotypes not detected involving different capacity of adhesion and internalization.

- <u>Normal primary bronchial/tracheal epithelial cells</u>

The infection of bronchial and tracheal epithelial human cell line (BTEC) with the invasive GAP58 strain and the SDSD isolates gave rise to a similar pattern of adherence and internalization as observed in Detroit 562 (9.36% for VSD21, 2.0% for VSD23 and 0% for VSD24 vs. 25.74% for GAP58) (Figure 7). However, the percentage of adherence and internalization was higher for both GAP58 and SDSD strains (Figure 4). Interestingly, VSD21, the isolate without any *S. pyogenes* virulence genes detected,

showed a higher percentage of adherence and internalization than VSD23 isolate (*sdn*) (Figure 7).

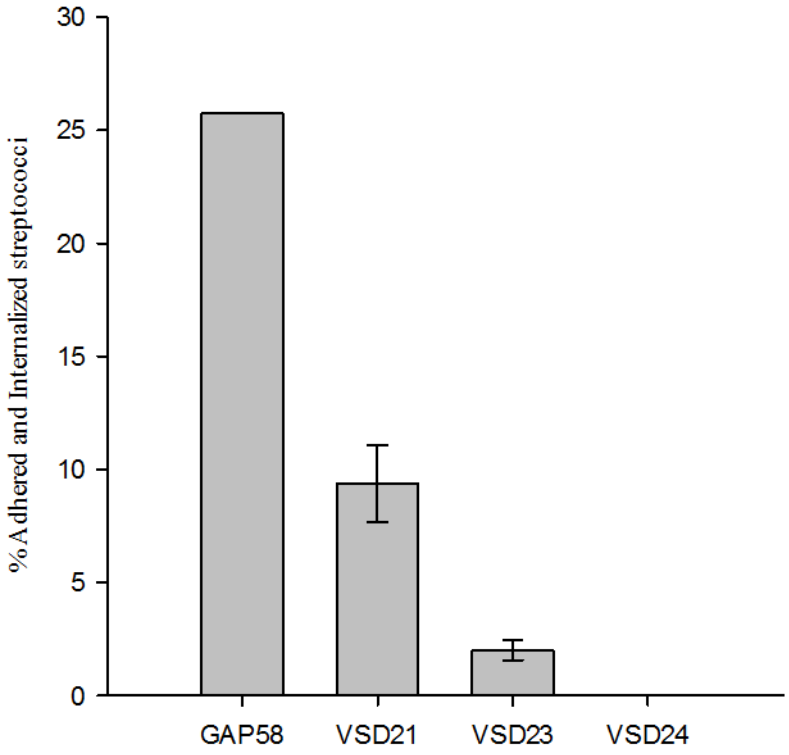

Figure 7. Percentage of adhered and internalized (interaction) streptococci on bronchial and tracheal epithelial human cell line. VSD21 – SDSD isolate without virulence genes detected (2 assays); VSD23 – SDSD isolate with one virulence gene (*sdn*) detected (2 assays); VSD24 – SDSD isolate with three virulence genes (*speC*, *speK*, *spd1*) detected (0 assays); GAP58 – *S. pyogenes* invasive strain isolated from human blood (1 assay). Statistical analysis could not be performed since GAP58 control strain had fewer than two valid treatment groups (number of assays considered) for Student's t-test method.

VSD24, the isolate with the most *S. pyogenes* virulence genes detected (*speC*, *speK*, *spd1*), did not show percentage of adherence and internalization due to the impossibility of recovering bacteria from the supernatant of the 96-well culture plates, after 2 h of incubation at 37ºC. Possible bacterial growth throughout the duration of assay could not be assessed and therefore, percentage of adhered and internalized streptococci could not be determined. This might be justified if non-adhered and non-internalized bacteria died

during or after the 2 h of incubation, since there were obtained adherent and internalized bacterial cells.

As for GAP58 strain, only one assay could be considered since the same happened as with VSD24, already discussed. These observations, particularly in the GAP58 control strain, impossibilities statistical analysis of SDSD infectious potential in BTEC since only one assay means that there are fewer than two valid treatment groups for Student's t-test method.

Since statistical analysis cannot be performed, the role of the *S. pyogenes* virulence genes, screened in this thesis, also cannot be fully discussed in relation with BTEC. Despite this, the higher percentage of adherence and internalization of VSD21 seems to point out that the virulence genes under study may not correlate with *in vitro* human respiratory tract cell infection potential.

SDSD strains belonging to the 2002-2003 collection (Table 11 – section 6. Annex) were shown to adhere to and internalize into human cell lines, but similarly, in a virulence gene independent manner. Indeed, VSD9, without *S. pyogenes* virulence genes detected, and VSD13, with 5 virulence genes (*speC, speK, speL, speM, spd1)* managed to interacted with BTEC in a similar manner compared to GAP58 strain, since the percentage of adherence and internalization was not significantly different than this control ($p > 0.05$), with the exception of VSD5 strain ($p = 0.007$) .

Together these results suggest that, despite the previous observation that the overall *S. pyogenes* virulence gene proportion was maintained throughout the years by strains of bovine *S. dysgalactiae* subsp. *dysgalactiae* species, the infection potential varies among strains independently of the virulence genes present. More studies are needed to identify other factors responsible for the ability of some SDSD isolates to infect human cells.

3.5. *In vivo* Zebrafish infection assay

- Contemporary *S. dysgalactiae* subsp. *dysgalactiae* zebrafish infection

Out of the 7 injected zebrafish with bovine *S. dysgalactiae* subsp. *dysgalactiae* VSD24 isolate (*speC*, *speK*, *spd1*), 30% (n=2) died during the 15 days of assay and the rest stayed healthy throughout it (70% survival rate). The results are shown in Table 9.
Both deaths occurred in the first 20 hours post-injection. The homogenization of the viscera and muscle of one fish showed a mixed bacterial growth (with α- and β-haemolytic bacteria). The second fish also displayed similar bacterial growth from the viscera, but not from the muscle where there was no growth observed (Table 9).

Bacterial identification, carried out by colony morphology, type of haemolysis in blood agar plates and Lancefield grouping, in both homogenates, allowed the identification of group C streptococci (GCS) in the first described fish. For the second fish, Lancefield grouping was not possible (Table 9).

Table 9. I*n vivo* infection of *Streptococcus dysgalactiae* subsp. *dysgalactiae* VSD24 and *S. pyogenes* invasive GAP58 strains in zebrafish

Injected strain code	Fish weight (mg)	Fish time of death (h)	Bacterial Peritoneal cavity infection (P)	Bacterial Intramuscular infection (M)	Bacterial Identification (P/M)
VSD24	260	20	mixed α- and β-	mixed α- and β-	GCS/GCS
VSD24	250	-	mixed α- and β-	-	GCS/-
VSD24	250	-	mixed α- and β-	-	GCS/-
VSD24	360	20	mixed α- and β-	-	N/I
VSD24	430	-	mixed α- and β-	-	Ongoing
VSD24	330	-	mixed α- and β-	-	Ongoing
VSD24	490	-	mixed α- and β-	-	Ongoing
GAP58	400	20	mixed α- and β-	pure	GAS/GAS
GAP58	250	20	mixed α- and β-	pure α-	GAS/GAS
GAP58	250	20	mixed α- and β-	mixed α-	GAS/GAS
GAP58	510	20	mixed α- and β-	mixed α- and β-	GAS/GAS
GAP58	290	20	mixed α- and β-	pure α-	GAS/GAS
GAP58	300	20	mixed α- and β-	pure β-	N/I
GAP58	160	20	mixed α- and β-	pure β-	GAS/GAS
GAP58	330	20	mixed α- and β-	pure β-	GAS/GAS
GAP58	200	20	mixed α- and β-	-	GAS/-

GCS – Lancefield Group C *Streptococcus*; GAS – Lancefield Group A *Streptococcus*; N/I – Not identified; α- – α-haemolysis; β- – β-haemolysis.

For two out of the five surviving zebrafish, GCS was isolated from the homogenization of the viscera and, similarly to the rest of the surviving fish, no bacterial growth was seen from the homogenization of the muscle. Despite this, all five surviving zebrafish displayed mixed α- and β-haemolytic bacterial growth from the viscera.

Bacterial identification needs to be concluded for the remaining three VSD24 injected fish visceral homogenization (inexistence of bacterial growth from the muscle homogenization).

This result demonstrates that although this SDSD isolate demonstrated the ability to cause an infection in fish (70% survival rate), there was not a statistical significant difference when compared to control zebrafish group injected with sterile culture medium (in which no zebrafish died; 100% survival). In the VSD24 case, there was observed heterogeneity in the infection response of this particular isolate since the fish that died post-injection without bacterial growth detected from in the muscle homogenization did not show any visual signs of disease (Figure 8). Variability of

individual host immune response might explain this observation since all zebrafish are wild-type. The same applies for the surviving fish.

Furthermore, despite the isolation of α-haemolytic bacteria from the visceral tissue of this asymptomatic fish, no bacterial growth was obtained from the homogenization of the caudal muscle. This suggests that, if this *S. dysgalactiae* subsp. *dysgalactiae* isolate was the cause of death, the infection was circumscribed and did not spread to sterile tissues and thus was not systemic. Alternatively, this fish might have died due to the injection procedure.

Figure 8. Zebrafish that died at 24 hours post-injection with *S. dysgalactiae* subsp. *dysgalactiae* VSD24 isolate showing no signs of disease. Image provided by C. Alves-Barroco.

Except for this example, for the other zebrafish that died after injection of this strain it was possible to isolate α-haemolytic bacteria as well as β-haemolytic from the muscle. This indicates that a systemic infection occurred in this case and that, probably, tissue necrosis and damage lead to the spread of other bacteria to this tissue, such as β-haemolytic bacteria which were always found in the visceral content of both dead zebrafish. This is explained by the presence of β-haemolytic gut bacteria such as *Aeromonas hydrophila, Pseudomonas aeroginosa, Vibrio parahaemolyticus and other,* which can act as opportunistic commensal organisms in zebrafish (Cantas *et al.*, 2012). It also explains why the 5 remaining surviving fish also had β-haemolytic bacterial growth isolated from the viscera.

Out of the 9 injected zebrafish with human *S. pyogenes* GAP58 invasive strain (*speA, speB, speF, speJ, smeZ*) it was observed focal and gross infection caused by this isolate and death observed in all injected fish at the first day post-injection which translates to 0% survival at the end of the assays (Table 9 and figure 9). The high death rate might be

explained by the nature of this isolate since it is an invasive strain isolated from the blood of a septicaemia human patient.

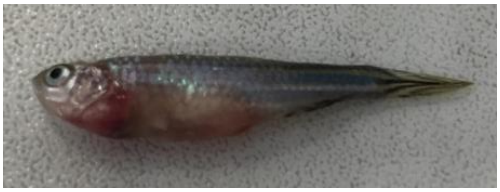

Figure 9. Representative zebrafish that died at 24 hours post-injection with *S. pyogenes* GAP58 invasive strain showing signs of disease. Image provided by C. Alves-Barroco.

For all GAP58 injected fish, both mixed α- and β-haemolytic bacterial growth was observed from visceral tissue, as also observed in VSD24 injected fish. All of these, with the exception of 1 fish, were identified as GAS (Table 9). As for the muscle tissue, 3 (out of the total 9) displayed pure β-haemolytic bacterial growth also identified as GAS, except the zebrafish already mentioned whose Lancefield grouping was not possible, and another with mixed α- and β-haemolytic colony growth also identified as GAS (Table 9).

Interestingly, 4 zebrafish did not display β-haemolytic colony growth from the muscle, but instead 2 fish displayed pure α-haemolytic, 1 fish mixed α-haemolytic and the fourth no haemolysis. GAS was identified in all these four cases. This could possibly be associated with the bacterial incubation conditions.

The isolation of α-haemolytic GCS bacteria from the muscle of the fish that died post-VSD24 injection and the inability of isolation of this serotype in GAP58 injected fish indicates that SDSD is the most probable cause of death in the reported fish. In addition, β-haemolytic and/or GAS isolation from this sterile site also points towards *S. pyogenes* infection in the case of GAP58 strain.

Statistical analysis showed that the infection of VSD24 isolate was statistically significantly different ($p \leq 0.05$) than GAP58 but not control injected zebrafish (p = 0.0183 and 0.129, respectively), which indicates that this SDSD isolate cannot induce infection of fish in the same manner as the *S. pyogenes* GAP58 invasive strain (Table 6

and Figure 9). Therefore both fish that died upon VSD24 isolate injection might have done so sporadically in accordance, as already mentioned above, with their particular immune response to the injection.

Moreover, the microbiological analysis of the viscera which allowed to detect the presence of α-haemolytic GCS bacteria in 2 out of 5 injected zebrafish that were euthanized after 15 days, suggesting a possible colonization ability of the bovine *S. dysgalactiae* subsp. *dysgalactiae* strains in these fish.

- Comparative zebrafish infection by *S. dysgalactiae* subsp. *dysgalactiae*

Figure 10 illustrates zebrafish survival analysis curves of both infection controls, medium injected and GAP58 strain injected, as well as the above discussed VSD24 strain isolate from the contemporary collection isolated between 2011-2013. In collaboration with C. Roma-Rodrigues and C. Alves-Barroco, the infection potential of 2 other *S. dysgalactiae* subsp. *dysgalactiae* strains in zebrafish was assessed and compared. Strains VSD9 (without *S. pyogenes* virulence genes detected) and VSD13 (*speC, speK, speL, speM, spd1*) were selected based on their *S. pyogenes* virulence gene profile, from the 2002-2003 collection characterized by Rato et al. (2010). All SDSD strains were studied in parallel and controls of infection were the same as for VSD24.

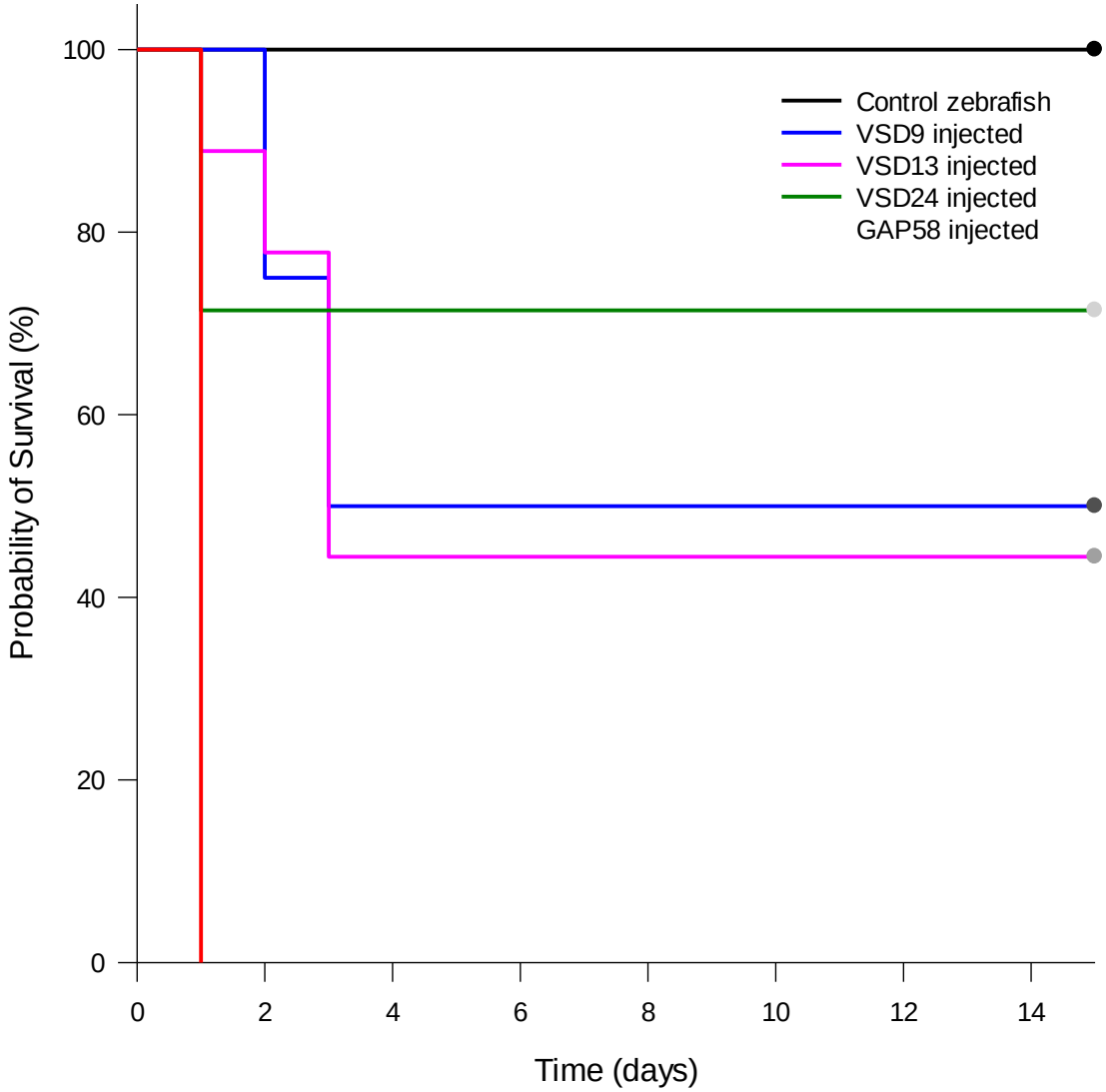

Figure 10. Zebrafish survival analysis curves with and without (control; sterile medium injection) SDSD VSD24 and *S. pyogenes* GAP58 strain infection. Survival probability throughout the 15 days of assay (lines) and censored surviving fish (dots) are depicted. Control zebrafish (black line), VSD9 injected zebrafish (blue line), VSD13 injected zebrafish (pink line), VSD24 injected zebrafish (green line) and GAP58 injected (red line) are depicted. There is a statistically significant difference between survival curves (p = < 0,001). Survival curves of all strains, with exception of VSD24 (p > 0.05), are statistically significantly different than the Control zebrafish survival curve.

Out of the 4 fish injected with *S. dysgalactiae* subsp. *dysgalactiae* VSD9 strain it was observed 2 fish deaths (in the first three days post-injection. After this period, fish managed to overcome a possible SDSD infection. None of these 2 fish died at the first day of assay.

As for SDSD VSD13 strain, 9 fish were injected. Similarly to VSD9 strain, there were no reported deaths past three days post-injection. During this period fish died every day, with the majority reported at the third day. In total, 56% (n=5) of VSD13 injected fish died (44% survival rate)

Statistical analysis revealed that the infection of VSD9 and VSD13 strains was statistically significantly different ($p < 0.05$) than GAP58 and control injected zebrafish (VSD9 $p = 0.00425$ and 0.0164, respectively for both controls, and VSD13 $p = 0.00203$ and 0.00827, also respective to both controls). Results indicate that these *S. dysgalactiae* subsp. *dysgalactiae* strains can indeed induce a higher infection rate in fish compared with control fish, and lower when compared to the *S. pyogenes* GAP58 invasive strain (Table 10 and Figure 10).

Altogether, the results obtained with the three *S. dysgalactiae* subsp. *dysgalactiae* isolates studied (VSD9, VSD13 and VSD24) confirms that this species can infect different hosts, namely bovines and fish. The fact that not all strains displayed the same ability implies that the infection potential is strain dependent.

S. dysgalactiae subsp. *dysgalactiae* VSD24, with three *S. pyogenes* virulence genes encoding SpeC and SpeK pyrogenic exotoxins and Spd1 DNase, infected fish with no statistical significant difference compared do control fish (Table 10). On the other hand VSD9, without any of these genes, and VSD13, with the most genes detected, could do so, although with less severity than GAP58. This suggests that these particular virulence genes screened by PCR, did not influence bacterial infection, and thus, infection potential in zebrafish is independent of these particular *S. pyogenes* virulence genes. This was also observed in the *in vitro* human cell line infections assays, discussed in section 3.4.

Table 10. Zebrafish infection assay statistical analysis comparison between *S. dysgalactiae* subsp. *dysgalactiae* and sterile medium, and *S. pyogenes* GAP58 infection controls. All pairwise multiple comparison procedures done with Holm-Sidak method ($p < 0.05$).

Comparisons	P Value	Significance
VSD9 vs. Control	0,0164	Yes
VSD9 vs. GAP58	0,00425	Yes
VSD13 vs. Control	0,00827	Yes
VSD13 vs. GAP58	0,00203	Yes
VSD24 vs. Control	0,129	No
VSD24 vs. GAP58	0,0183	Yes

4. Conclusions

This work aimed at evaluating if carriage of *S. pyogenes* phage virulence genes is shared by strains of the strictly animal pathogen *S. dysgalactiae* subsp. *dysgalactiae* isolated in 2011-2013 in dairy herds in Portugal, and assessing if that property if species-specific by comparison with a previous group of *S. dysgalactiae* subsp. *dysgalactiae* strains, isolated in 2002-2003 and characterized by Marcia *et al*. (2010). It also aimed at evaluating if these virulence genes can influence the virulence potential of strains of this species using *in vitro* and in *vivo* studies to infer a zoonotic potential.

Our main conclusions and remarks are the following:

- Carriage of virulence factors, such as superantigens and DNases, encoded by phages of the strictly human pathogen *S. pyogenes* phage are most probably a characteristic of *S. dysgalactiae* subsp. *dysgalactiae* of bovine origin.

- *S. dysgalactiae* subsp. *dysgalactiae* isolates collected in 2011-2013 produce extracellular DNases, independently on the presence and expression of *spd1* and *sdn* genes.

- *S. dysgalactiae* subsp. *dysgalactiae* isolates collected in 2011-2013 could not adhere and internalize into the pharyngeal carcinoma epithelial human cells Detroit562, contrary to the control *S. pyogenes* human invasive strain and to other *S. dysgalactiae* subsp. *dysgalactiae* isolates from the 2002-2003 collection.

- Adherence and internalization of *S. dysgalactiae* subsp. *dysgalactiae* isolates collected in 2011-2013 to bronchial and tracheal epithelial human cell line could not be statistically assessed. Two bovine isolates from the 2002-2003 collection could indeed interact with this cell line in a similar way as control *S. pyogenes* human invasive strain.

- *S. dysgalactiae* subsp. *dysgalactiae* VSD21 (isolate from the 2011-2013 collection, without *S. pyogenes* virulence genes) could interact more with both human cell lines

than other contemporary bovine isolates, similarly with VSD9 strain (from 2002-2003 collection). Therefore, the results suggest that *S. pyogenes* phage virulence gene frequency does not correlate with *in vitro* infectious potential of these strains.

- Zebrafish intraperitoneally injection of *S. dysgalactiae* subsp. *dysgalactiae* strains revealed that not all bovine isolates can infect this animal model in the same manner. Infection potential varies between strain and is not dependent on the *S. pyogenes* phage virulence genes profiled.

This thesis helped to answer to some questions regarding the role of the genotype of *S. dysgalactiae* subsp. *dysgalactiae*, regarding *S. pyogenes* virulence determinants, in the infection potential in human respiratory cell lines and in the animal model, zebrafish. As these isolates were collected from cases of bovine mastitis from dairy farms, possible transmission to humans is of major concern**.**

In order to continue this work and to confirm the results obtained:

- Perform at least 3 new *in vitro* infection assays using the normal primary bronchial/tracheal epithelial cell line.

- Conduct new *in vitro* and *in vivo* infection assays with bacterial isolates from the same collections with different genotypes.

- Study other *in vitro* cell lineages such as bovine and human primary epithelial cells from the mammary gland.

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6. Annex

Table 11. *Streptococcus dysgalactiae* subp. *dysgalactiae* isolates from the 2002-2003 collection chosen for the *in vitro* infection assays based on their Group A *S. pyogenes* (GAS) virulence genes detected.

Species	Strain code	GAS virulence genes detected	Clinical origin	Ref.
Streptococcus dysgalactiae subsp. *dysgalactiae*	VSD5	*sdn*	Subclinical	Rato *et al*., 2010
	VSD9	none	Subclinical	
	VSD13	*speC, speK, speL, speM, spd1*	Subclinical	

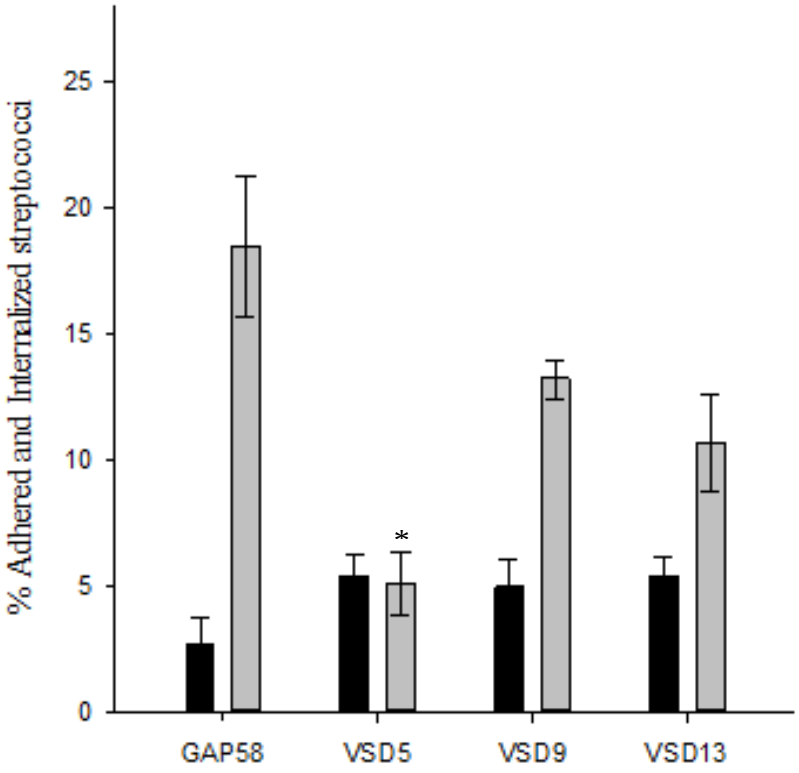

Figure 11. Percentage of adhered and internalized (interaction) streptococci on Detroit 562 (black bars) and bronchial and tracheal epithelial human cell line (grey bars). VSD5 - SDSD strain with one virulence gene (*sdn*) detected (P = 0.062 for Detroit562 and P = 0.007 for BTEC); VSD9 - SDSD strain without virulence genes detected (P = 0.073 for Detroit562 and P = 0.843 for BTEC); VSD13 - SDSD strain with five virulence genes (*speC, speK, speL, speM, spd1*) detected (P = 0.065 for Detroit562 and P = 0.122 for BTEC); GAP58 – SP invasive strain isolated from human blood. * – *p* value ≤ 0.05. Statistical group comparison was performed using Student's t-test method. Results provided by C. Roma-Rodrigues and C. Alves-Barroco.